

# WEST Search History

DATE: Sunday, August 10, 2003

**Set Name Query**

side by side

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result set

*DB=USPT; PLUR=YES; OP=OR*

L7 5294441.pn.

1 L7

*DB=USPT,PGPB; PLUR=YES; OP=OR*

L6 l3 and pressure

3 L6

L5 ((pressure/ )!.CCLS. |(and/ )!.CCLS. |(l3/ )!.CCLS. )

0 L5

L4 L3 and mexicana

11 L4

L3 L2 and lysate

30 L3

L2 L1 and leishmania

54 L2

L1 ((424/269.1 )!.CCLS. )

130 L1

END OF SEARCH HISTORY

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☐ 11. [5834592](#). 22 Sep 95; 10 Nov 98. Leishmania antigens for use in the therapy and diagnosis of Leishmaniasis. Reed; Steven G., et al. 530/350; 424/184.1 [424/269.1](#) 530/364 530/806 930/210. C07K001/00 C07K014/00 C07K017/00 A61K039/002.

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L3 and mexicana	11

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- ☐ 1. [20020081320](#). 04 Jun 01. 27 Jun 02. Leishmania antigens for use in the therapy and diagnosis of leishmaniasis. Reed, Steven G., et al. [424/269.1](#); 435/183 435/252.33 435/325 514/44 A61K039/008 A61K048/00 C12N009/00 C12N001/21 C12N005/06.
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- ☐ 2. [6500437](#). 14 Apr 00; 31 Dec 02. Leishmania antigens for use in the therapy and diagnosis of leishmaniasis. Reed; Steven G., et al. [424/269.1](#); 424/184.1 424/191.1 424/192.1 424/265.1 424/85.2 435/69.7 514/12 514/2 514/44 530/300 530/350 536/23.1 536/23.4. A61K039/008 A61K039/00 A61K038/00 A61K039/002 A61K045/00.
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- ☐ 3. [6491922](#). 04 Feb 97; 10 Dec 02. Methods and compounds for treating autoimmune and vascular disease. Ho; John L.. 424/193.1; 424/265.1 [424/269.1](#) 514/2. A61K039/385 A61K039/00 A61K039/002 A61K038/00.
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- ☐ 4. [6410250](#). 29 Nov 99; 25 Jun 02. Sustained delivery device and methods of making and using the same. Gueiros-Filho; Frederico J., et al. 435/7.22; [424/269.1](#) 435/455 536/23.1 536/23.2. G01N033/53 G01N033/569 C12N015/63 A61K039/002 C07H021/02.
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- ☐ 5. [6403103](#). 10 Dec 97; 11 Jun 02. Trypanosoma cruzi antigen, gene encoding therefore, and methods of detecting and treating chagas disease. Paranhos-Baccala; Glaucia, et al. [424/269.1](#); 424/185.1 424/190.1 424/193.1 424/265.1 435/7.1 530/350 530/387.1 530/387.2 530/387.9 530/388.6. A61K039/002.
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- ☐ 6. [6375955](#). 12 Feb 98; 23 Apr 02. Leishmania antigens for use in the therapy and diagnosis of leishmaniasis. Reed; Steven G., et al. [424/269.1](#); 424/184.1 424/265.1 530/300 530/350 930/210. A61K039/008 A61K039/00 A61K038/00.
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- ☐ 7. [6365165](#). 30 Oct 98; 02 Apr 02. Leishmania antigens for use in the therapy and diagnosis of Leishmaniasis. Reed; Steven G., et al. [424/269.1](#); 424/184.1 424/265.1 424/450 424/85.2 514/12 514/2 514/44. A61K039/008 A61K039/00 A61K045/00 A61K038/00 A61K039/002.
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- ☐ 8. [6020144](#). 23 Oct 96; 01 Feb 00. Sustained delivery device comprising a Leishmania protozoa and methods of making and using the same. Gueiros-Filho; Frederico J., et al. 435/7.22; [424/269.1](#) 424/94.3 435/245 435/258.3 435/91.41 435/91.42. G01N033/53 C12N015/64 C12N001/36 C12N001/10.
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- ☐ 9. [5965143](#). 20 Jun 96; 12 Oct 99. Immunity to trypanosomatids species. Fasel; Nicolas Joseph, et al. [424/269.1](#); 435/252.33 435/320.1 435/6 514/44 536/23.4 536/23.5. A61K039/008 A61K048/00 C12Q001/68 C07H021/00.
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- ☐ 10. [5965142](#). 04 Aug 95; 12 Oct 99. Polypeptides and methods for the detection of L. tropica infection. Dillon; Davin C., et al. [424/269.1](#); 424/184.1 424/268.1 530/300 530/350. A61K038/00 A61K039/002 C07K002/00 C07K014/00.
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L3 and mexicana	11

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- ☐ 11. [6228372](#). 18 Dec 97; 08 May 01. Compounds and methods for the detection and prevention of *T. cruzi* infection. Reed; Steven G., et al. [424/269.1](#); [424/185.1](#) [424/191.1](#) [424/192.1](#) [424/193.1](#) [424/200.1](#) [435/4](#) [435/69.1](#) [435/69.3](#) [435/69.7](#) [435/7.1](#) [435/7.22](#) [435/7.4](#) [435/7.92](#) [435/71.1](#) [530/300](#) [530/324](#) [530/333](#) [530/334](#) [530/344](#) [530/350](#) [530/403](#) [530/412](#). A61K039/002.
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- ☐ 12. [6207158](#). 05 Aug 97; 27 Mar 01. Parasitic helminth macrophage migration inhibitory factor proteins, nucleic acid molecules, and uses thereof. Tripp; Cynthia Ann, et al. [424/184.1](#); [424/185.1](#) [424/191.1](#) [424/265.1](#) [424/269.1](#) [435/6](#) [435/975](#) [530/300](#) [530/350](#). A61K039/00 A61K039/002 C12Q001/68 C07K001/00.
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- ☐ 13. [6133017](#). 02 Jun 95; 17 Oct 00. Attenuated strain of *Leishmania*. Matlashewski; Gregory, et al. [435/258.3](#); [424/269.1](#). C12N001/10.
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- ☐ 14. [6054135](#). 15 Apr 97; 25 Apr 00. Compounds and methods for the detection and prevention of *T. cruzi* infection. Reed; Steven G., et al. [424/269.1](#); [424/185.1](#) [424/191.1](#) [424/192.1](#) [424/193.1](#) [530/350](#). A61K039/002.
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- ☐ 15. [6020144](#). 23 Oct 96; 01 Feb 00. Sustained delivery device comprising a *Leishmania* protozoa and methods of making and using the same. Gueiros-Filho; Frederico J., et al. [435/7.22](#); [424/269.1](#) [424/94.3](#) [435/245](#) [435/258.3](#) [435/91.41](#) [435/91.42](#). G01N033/53 C12N015/64 C12N001/36 C12N001/10.
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- ☐ 16. [6013268](#). 12 Dec 97; 11 Jan 00. Methods for enhancement of protective immune responses. Reed; Steven G. [424/269.1](#); [424/184.1](#) [424/265.1](#) [424/450](#) [514/12](#) [514/44](#) [530/350](#) [536/23.1](#). A61K048/00 A61K039/00 A61K031/70 C07K014/00.
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- ☐ 17. [5965143](#). 20 Jun 96; 12 Oct 99. Immunity to trypanosomatids species. Fasel; Nicolas Joseph, et al. [424/269.1](#); [435/252.33](#) [435/320.1](#) [435/6](#) [514/44](#) [536/23.4](#) [536/23.5](#). A61K039/008 A61K048/00 C12Q001/68 C07H021/00.
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- ☐ 18. [5965142](#). 04 Aug 95; 12 Oct 99. Polypeptides and methods for the detection of *L. tropica* infection. Dillon; Davin C., et al. [424/269.1](#); [424/184.1](#) [424/268.1](#) [530/300](#) [530/350](#). A61K038/00 A61K039/002 C07K002/00 C07K014/00.
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- ☐ 19. [5958414](#). 03 Sep 97; 28 Sep 99. Composition to protect a mammal against *Bartonella henselae* infection. Regnery; Russell L., et al. [424/184.1](#); [424/269.1](#) [424/278.1](#) [424/280.1](#) [435/243](#). A61K039/00 A61K045/00 A61K039/002 C12N001/00.
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- ☐ 20. [5916572](#). 14 Nov 95; 29 Jun 99. Compounds and methods for the detection and prevention of *T. cruzi* infection. Reed; Steven G., et al. [424/269.1](#); [424/190.1](#) [530/350](#) [530/388.6](#) [536/23.1](#). A61K039/002.
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- ☐ 21. [5910306](#). 14 Nov 96; 08 Jun 99. Transdermal delivery system for antigen. Alving; Carl R., et al. 424/184.1; 424/204.1 424/234.1 424/265.1 [424/269.1](#) 424/274.1 424/277.1 424/279.1 424/282.1 424/283.1 424/449 424/450 424/810 424/812. A61K039/00 A61K039/002 A61K039/02 A61K039/12.
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- ☐ 22. [5879687](#). 18 Apr 96; 09 Mar 99. Methods for enhancement of protective immune responses. Reed; Steven G.. [424/269.1](#); 424/184.1 514/12. A61K039/008 A61K039/39 C07K014/44 C12N015/30.
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- ☐ 11. [6228372](#). 18 Dec 97; 08 May 01. Compounds and methods for the detection and prevention of *T. cruzi* infection. Reed; Steven G., et al. [424/269.1](#); [424/185.1](#) [424/191.1](#) [424/192.1](#) [424/193.1](#) [424/200.1](#) [435/4](#) [435/69.1](#) [435/69.3](#) [435/69.7](#) [435/7.1](#) [435/7.22](#) [435/7.4](#) [435/7.92](#) [435/71.1](#) [530/300](#) [530/324](#) [530/333](#) [530/334](#) [530/344](#) [530/350](#) [530/403](#) [530/412](#). A61K039/002.
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- ☐ 13. [6133017](#). 02 Jun 95; 17 Oct 00. Attenuated strain of *Leishmania*. Matlashewski; Gregory, et al. [435/258.3](#); [424/269.1](#). C12N001/10.
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- ☐ 17. [5965143](#). 20 Jun 96; 12 Oct 99. Immunity to trypanosomatids species. Fasel; Nicolas Joseph, et al. [424/269.1](#); [435/252.33](#) [435/320.1](#) [435/6](#) [514/44](#) [536/23.4](#) [536/23.5](#). A61K039/008 A61K048/00 C12Q001/68 C07H021/00.
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- ☐ 20. [5916572](#). 14 Nov 95; 29 Jun 99. Compounds and methods for the detection and prevention of *T. cruzi* infection. Reed; Steven G., et al. [424/269.1](#); [424/190.1](#) [530/350](#) [530/388.6](#) [536/23.1](#). A61K039/002.
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- ☐ 7. [6410250](#). 29 Nov 99; 25 Jun 02. Sustained delivery device and methods of making and using the same. Gueiros-Filho; Frederico J., et al. 435/7.22; [424/269.1](#) 435/455 536/23.1 536/23.2. G01N033/53 G01N033/569 C12N015/63 A61K039/002 C07H021/02.
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- ☐ 8. [6403103](#). 10 Dec 97; 11 Jun 02. Trypanosoma cruzi antigen, gene encoding therefore, and methods of detecting and treating chagas disease. Paranhos-Baccala; Glaucia, et al. [424/269.1](#); [424/185.1](#) [424/190.1](#) [424/193.1](#) [424/265.1](#) 435/7.1 530/350 530/387.1 530/387.2 530/387.9 530/388.6. A61K039/002.
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- ☐ 9. [6375955](#). 12 Feb 98; 23 Apr 02. Leishmania antigens for use in the therapy and diagnosis of leishmaniasis. Reed; Steven G., et al. [424/269.1](#); [424/184.1](#) [424/265.1](#) 530/300 530/350 930/210. A61K039/008 A61K039/00 A61K038/00.
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- ☐ 1. 6410250. 29 Nov 99; 25 Jun 02. Sustained delivery device and methods of making and using the same. Gueiros-Filho; Frederico J., et al. 435/7.22; 424/269.1 435/455 536/23.1 536/23.2. G01N033/53 G01N033/569 C12N015/63 A61K039/002 C07H021/02.
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- ☐ 2. 6020144. 23 Oct 96; 01 Feb 00. Sustained delivery device comprising a Leishmania protozoa and methods of making and using the same. Gueiros-Filho; Frederico J., et al. 435/7.22; 424/269.1 424/94.3 435/245 435/258.3 435/91.41 435/91.42. G01N033/53 C12N015/64 C12N001/36 C12N001/10.
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- ☐ 3. 5910306. 14 Nov 96; 08 Jun 99. Transdermal delivery system for antigen. Alving; Carl R., et al. 424/184.1; 424/204.1 424/234.1 424/265.1 424/269.1 424/274.1 424/277.1 424/279.1 424/282.1 424/283.1 424/449 424/450 424/810 424/812. A61K039/00 A61K039/002 A61K039/02 A61K039/12.
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## WEST Search History

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L3	6284739	1	L3
L2	6433023	1	L2
L1	5202320	5	L1

END OF SEARCH HISTORY

**End of Result Set**☐ **Generate Collection**

L1: Entry 5 of 5

File: USPT

Apr 13, 1993

US-PAT-NO: 5202320DOCUMENT-IDENTIFIER: US 5202320 A

TITLE: Method for treating leishmaniasis

DATE-ISSUED: April 13, 1993

## INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Tidwell; Richard R.	Chapel Hill	NC	27514	
Geratz; J. Dieter	Chapel Hill	NC	27514	
Hall; James E.	Chapel	NC	27514	
Kyle; Dennis E.	Silver Spring	MD	20901	
Grogl; Max	Brookville	MD	20833	
Ohemeng; Kwasi A.	Clinton	NJ	08809	

APPL-NO: 07/ 334730 [PALM]

DATE FILED: April 6, 1989

INT-CL: [05] A61K 31/55, A61K 31/505, A61K 31/415, A61K 31/155

US-CL-ISSUED: 514/218; 514/256, 514/402, 514/636

US-CL-CURRENT: 514/218; 514/256, 514/402, 514/636

FIELD-OF-SEARCH: 514/636, 514/218, 514/256, 514/402

PRIOR-ART-DISCLOSED:

## OTHER PUBLICATIONS

Reviews of Infectious Disease, vol. 10, No. 3 pp. 576-577.  
Experimental Parasitology, vol 52, p. 404-413 (1981).

ART-UNIT: 125

PRIMARY-EXAMINER: Goldberg; Jerome D.

ATTY-AGENT-FIRM: Drehkoff; W. Dennis

## ABSTRACT:

This invention relates to a method for treating leishmaniasis which comprises administering to an afflicted host patient, a therapeutically effective amount of a compound having the following structure; ##STR1## wherein X is O or NH; R.sub.1 is H or two R.sub.1 of the same amidine group together represent --(CH.sub.2).sub.m -- wherein m=2, 3 or 4; R.sub.2 is H, NH.sub.2, OCH.sub.3, Cl or NO.sub.2 ; R.sub.3 is H, CH.sub.3 or CH.sub.2 CH.sub.3 and n=2-6 or a pharmaceutical acceptable salt thereof provided that X is O both R.sub.2 and both R.sub.3 cannot be H.

1 Claims, 0 Drawing figures

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- ☐ 1. 6326395. 15 Sep 99; 04 Dec 01. Antifungal activity of dicationic molecules. Tidwell; Richard R., et al. 514/461; 549/200 549/210. A61K031/34 C07D321/00 C07D307/00.
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- ☐ 3. 5455306. 28 Feb 94; 03 Oct 95. Aqueous coating composition. Mori; Yoshio, et al. 525/303; 525/295 525/301 525/305. C08F225/04.
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- ☐ 5. 5202320. 06 Apr 89; 13 Apr 93. Method for treating leishmaniasis. Tidwell; Richard R., et al. 514/218; 514/256 514/402 514/636. A61K031/55 A61K031/505 A61K031/415 A61K031/155.

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L2: Entry 1 of 1

File: USPT

Aug 13, 2002

US-PAT-NO: 6433023DOCUMENT-IDENTIFIER: US 6433023 B1

TITLE: Compositions having anti-leishmanial activity

DATE-ISSUED: August 13, 2002

## INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Callahan; Heather	Lajolla	CA		
Kelley; Colleen	Flagstaff	AZ		
Grogl; Max	Columbia	MD		
Schuster; Brian G.	Vienna	VA		

US-CL-CURRENT: 514/706; 514/741

## CLAIMS:

What we claim is:

1. A pharmaceutical composition of matter comprising an antiparasitic effective amount of a compound of the formula I ##STR2## wherein R.sub.4 is a leaving group selected from the group consisting of chloro, alkoxy, bromo, an amino which forms a primary or secondary amine, hydroxy and thiol; R.sub.3 and R.sub.5 are selected from the group consisting of H, nitro, amino, CF.sub.3, carboxy and a sulfur containing group, wherein at least one of R.sub.3 and R.sub.5 is nitro, in a pharmaceutically acceptable carrier.
2. A composition of claim 1 wherein, in formula I, R.sub.4 is Cl and one of R.sub.3 and R.sub.5 is nitro and the other of R.sub.3 and R.sub.5 is nitro or a sulfur-containing group.
3. A composition of claim 1 wherein R.sub.4 is Cl and R.sub.3 and R.sub.5 are both nitro.
4. A composition of claim 2 wherein one of R.sub.3 and R.sub.5 is thiol.
5. A composition of claim 2 wherein one of R.sub.3 and R.sub.5 is sulfonyl.
6. A composition of claim 1 wherein R.sub.4 is bromo.
7. A method of inhibiting growth of a parasite having microtubules in a person infected with said parasite comprising administration of an antiparasitic effective amount of the composition of claim 1.
8. A method of claim 7 wherein, in formula I, R.sub.4 is Cl and one of R.sub.3 and R.sub.5 is nitro and the other of R.sub.3 and R.sub.5 is nitro or a sulfur-containing group.
9. A method of claim 8 wherein, in formula I, R.sub.4 is Cl and R.sub.3 and R.sub.5 are both nitro.
10. A method of claim 8 wherein, in formula I, one of R.sub.3 and R.sub.5 is

thiol.

11. A method of claim 8 wherein, in formula I, one of R.sub.3 and R.sub.5 is sulfonyl.
12. A method of claim 7 wherein the composition is administered parenterally.
13. A method of claim 7 wherein the composition is administered orally.
14. A method of claim 7 wherein the composition is administered topically.
15. A method of claim 7 wherein the composition is administered transdermally.

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- ☐ 1. 6433023. 26 Mar 98; 13 Aug 02. Compositions having anti-leishmanial activity. Callahan; Heather, et al. 514/706; 514/741. A61K031/095.
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L3: Entry 1 of 1

File: USPT

Sep 4, 2001

US-PAT-NO: 6284739DOCUMENT-IDENTIFIER: US 6284739 B1

TITLE: Antileishmanial composition for topical application

DATE-ISSUED: September 4, 2001

## INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Grogli, Max	Brookeville	MD	20853	
Fleckenstein, Lawrence	Iowa City	IA	52246	
McGreevy, Patrick	Silver Spring	MD	20902	
Schuster, Brian	Vienna	VA	22182	

US-CL-CURRENT: 514/40; 514/38, 514/39

## CLAIMS:

What is claimed is:

1. A leishmania-inhibiting pharmaceutical composition of matter comprising an antileishmania-effective amount of paromomycin of 10% to 40% or a pharmaceutically acceptable salt thereof and an paromomycin-potentiating effective amount of gentamicin of 0.2% to 5%, or a pharmaceutically acceptable salt thereof in a pharmaceutically effective carrier.
2. A composition of claim 1 in a emulsion base.
3. A composition of claim 1 wherein the carrier contains alcohols, non-toxic glycols and petrolatum.
4. A composition of claim 1 further containing a penetrant.
5. A composition of claim 4 wherein the penetrant is urea.
6. A composition of claim 1 further containing a preservative.
7. A composition of claim 3 containing urea and, additionally, sorbitol, propylene glycol, lactic acid, sodium lauryl sulfate, isopropyl palmitate, stearyl alcohol, white petrolatum, propyl paraben, and methyl paraben.
8. A composition of claim 7 containing 15% paromomycin sulfate and 0.5% gentamicin sulfate.
9. A method of ameliorating the effect of leishmaniasis comprising administration of a composition of claim 1 to a patient suffering from leishmaniasis.
10. A method of claim 9 wherein the leishmaniasis results from infection with a New World strain of Leishmania.
11. A method of claim 9 wherein the leishmaniasis results from infection with an Old World strain of Leishmania.

12. A method of claim 9 wherein the composition of claim 1 is administered twice daily for at least two consecutive days.
13. A method of treating leishmaniasis comprising topical administration of a composition of claim 1 to a patient suffering from leishmaniasis.
14. A method of claim 9 wherein the composition is administered topically.



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L6: Entry 1 of 3

File: USPT

Jun 25, 2002

DOCUMENT-IDENTIFIER: US 6410250 B1

TITLE: Sustained delivery device and methods of making and using the same

Brief Summary Text (23):

As used herein, the term "organism" includes any unicellular organism suitable for use in the devices and methods of the present invention. Haploid and diploid, including asexual diploid, unicellular organisms are contemplated. Particularly preferred unicellular organisms include protozoa, especially parasitic protozoa. Set forth below is a non-limiting list of those unicellular protozoa contemplated to be within the scope of the present invention. Also set forth below is a discussion of generally preferred features and characteristics of the unicellular organisms most suitable for use in the present invention. As exemplified in Examples 2 and 8, respectively, two diploid genera currently preferred are *Leishmania* and *Trypanosoma*. Most currently preferred are the species *L. major*, *L. tropica*, *L. aethiopica*, *L. enrietti*, *L. panamaensis*, *L. guyanensis*, *L. donovani*, *L. chagasi*, *L. infantum*, *T. cruzi*, *T. brucei* and members of the trypanosomatid genus *Endotrypanum* such as *E. monterogeii* and *E. schaudinni*. As also exemplified in Example 8, a currently preferred haploid genus is *Toxoplasma*; a most currently preferred species is *T. gondii*. Other currently preferred haploid genera include *Plasmodium*, *Eimeria*, and *Cryptosporidia*. A currently preferred genus of uncertain ploidy is *Giardia*. A most currently preferred species is *G. lamblia*. Other preferred genera of uncertain ploidy include *Entamoeba*, *Acanthamoeba* and *Naegleria*, and *Microsporida* and *Trichomona* (*T. vaginalis* and *T. foetus*, in particular) generally.

Brief Summary Text (26):

Other embodiments of the present invention contemplate unicellular organisms rendered conditionally defective by interrupting, or otherwise disrupting, a naturally-occurring genetic locus. Methods of achieving such interruptions or disruptions using transposons are exemplified below in Example 4. Again, it is understood that the extent of, or precise manner of, physical interruption of a nucleotide sequence comprising a particular genetic locus need only be enough to confer on the organism a selectable phenotype. As used herein, the term "transposon" refers to a discreet genetic element that insures its own maintenance by inserting into other autonomously maintained genetic elements. Transposons can be constructed to further encode substances of particular interest, for example a heterologous gene encoding an expression product of therapeutic interest. Transposons are useful tools for genetic manipulations such as deletions, inversions, and fusions, as is well known in the art. A transposon is a specific DNA segment with the ability to move as a unit in more or less random fashion from one genetic locus to another. Exemplary of specific transposons which may be used herein are transposons originally derived from insects, preferably members of the *Tc1/mariner* family, most preferably the *mariner* transposon element from *Drosophila*. As disclosed herein in Example 4, insertional inactivation mediated by a transposon can be a powerful way to generate mutants and gene fusions which would facilitate studies of gene function in an asexual diploid like *Leishmania*. The development of a *mariner*-based heterologous transposon system is a significant addition to the array of tools available for dissecting the genetic basis of relevant aspects of *Leishmania* biology, such as virulence and pathogenesis. The same system can be developed to ameliorate the restrictions of other asexual diploids using methods within the scope of the present invention.

Brief Summary Text (35):

The following non-limiting examples of genes can be used as negative markers according to methods of the invention: adenine (APRT), hypoxanthine/guanine (HGPRT), hypoxanthine/guanine/xanthine (HXGPRT), uracil phosphoribosyl transferase (UPRT), dihydrofolate reductase-thymidine synthetase (DHFR-TS), thymidine kinase (TK) and orotidine 5'-phosphate-decarboxylase. These markers can be selected against using methods known in the art which illustrate some of the general principles of negative selection described above. A specific example of using DHFR-TS as a negative marker for null-targeting in *Leishmania* is provided in Example 2.

Brief Summary Text (44):

As exemplified herein in Example 5, the present invention also provides a device for delivering an immunostimulant to a mammal. In certain embodiments, this immunostimulating property of the instant device serves to vaccinate the recipient. Accordingly, the present invention can serve as an improvement for a conventional vaccine for the following diseases and/or conditions: pediatric pertussis, HIV infection, multiple sclerosis, melanoma, breast cancer, diarrheal diseases and gastritis, CMV virus, prostate and ovarian cancers, diphtheria, tetanus, cancers such as colorectal, stomach, and pancreatic, peptic ulcers, melanoma, hepatitis B, herpes simplex, influenza, psoriasis, lyme disease, infectious diseases generally, respiratory syncytial virus, and various protozoa and parasitic protozoa such as Leishmania, to name but a few.

Brief Summary Text (47):

Among the flagellate protozoa, there is a subgroup called kinetoplastids (trypanosomes and their allies). Most of the 600 or so species of kinetoplastids are parasites, including important genera such as Trypanosoma and Leishmania which parasitize vertebrates, including humans. These genera are among two of the currently preferred genera of diploid organisms suitable for practice with the instant invention. Other genera can be found as gut parasites in insects and as parasites in plants. Accordingly, members of such genera can be used to practice the instant invention when the desired host is an insect or a plant. Among those spore forming groups of protozoa, there are three subcategories. They include microspora, sporozoa, and mixospora. The microspora are intracellular parasites. There are a number of microspora genera which parasitize invertebrates such as Encephalitozoon in mammals, including humans with immune deficiency, and Glugea in fish. Another wide spread genera is Nosena, the species of which cause important diseases in insects of economic importance such as bees and silk worms. Again, in applications involving hosts such as non-human vertebrates or insects, the delivery device of the present invention can be exploited most successfully. Among the sporozoa category, there are numerous intestinal, blood, and tissue dwelling coccidians suitable for use with the present invention. Particularly useful coccidians include Cryptosporidium, Isospora, Toxoplasma, Sarcocystis, and Plasmodium. Certain species of genera of Eimeria and Isospora are important pathogens of domestic animals. Toxoplasma is a widely distributed parasite of mammals that is now regarded by certain artisans as a member of the genus Isospora. Toxoplasma is a currently preferred haploid genera suitable for use with the present invention. Finally, members of the subcategory mixospora include protozoa commonly considered parasitic of fish. For example, it is currently believed that a member of the mixospora category is responsible for "whirling disease" in trout. The skilled artisan will appreciate that the instant invention would be applicable to control such a disease.

Brief Summary Text (48):

While a generally useful feature of currently preferred organisms is that they can be cultured in vitro, this is not a requirement. Among the above-referenced diploid genera, two currently preferred are Leishmania and Trypanosoma, both blood and tissue flagellates reside in humans. Both genera can, in certain stages of their life cycle, be propagated in culture. Moreover, numerous aspects of the molecular biology of protein processing and expression have been studied in these genera. Features shared with higher eucaryotes include synthesis of capped polyadenylated cytoplasmic RNA; a typical ribosome and protein synthetic apparatus; and, a general protein secretory apparatus including endoplasmic reticulum, golgi apparatus and exo- and endo-cytosis.

Brief Summary Text (49):

In the genera Leishmania, several species can cause visceral disease and reside intracellularly, for example, in lymph nodes, liver, spleen, bone marrow, etc. Other species of Leishmania cause cutaneous and mucocutaneous diseases. Such species are found intracellularly and extracellularly in skin and mucous membranes of humans. Within the genera Trypanosoma, it is well known that certain species reside intracellularly in viscera, myocardium, and brain in humans. During certain stages of their developmental cycle, these species may also reside in blood, lymph nodes, cerebro-spinal fluid, depending upon whether the organism is residing in the host or the vector.

Brief Summary Text (51):

As already stated, two particularly preferred genera of protozoa include Leishmania and Toxoplasma. Another currently preferred genera is Giardia. In certain embodiments, other pathogenic amoebae are contemplated. Specifically, the currently preferred

unicellular diploid organisms include, but are not limited to: Protozoans of the family Trypanosomatidae (*Trypanosoma* *crúzi*, *T. brucei*, *Leishmania* spp (including subgenus *Leishmania* and *Viannia*; examples include *L. major*, *L. tropica*, *L. aethiopica*, *L. entrietti*, *L. mexicana*, *L. amazonensis*, *L. donovani*, *L. chagasi*, *L. infantum*, *L. braziliensis*, *L. panamaensis*, *L. guyanensis*, and others). The currently preferred haploid organisms include, but are not limited to: members of Apicomplexa (*Toxoplasma gondii*, *Plasmodium*, *Eimeria*, *Cryptosporidia*, and others). Protozoans of uncertain ploidy include, but are not limited to: amoebae including *Entamoeba* spp, *Acanthamoeba* spp, *Naegleria* spp, *Giardia lamblia*, and members of the phyla including Microsporida and the trichomonads (*Trichomonas vaginalis*, *Tritrichomonas foetus*).

#### Brief Summary Text (52):

Other suitable protozoans known to have human hosts include: *Entamoeba histolytica*, *Entamoeba hartmanni*, *Entamoeba coli*, *Entamoeba polecki*, *Endolimax nana*, *Iodamoeba buetschlii*, *Naegleria fowleri*, *Acanthamoeba* species, *Dientamoeba fragilis*, *Giardia lamblia*, *Chilomastix mesnili*, *Trichomonas vaginalis*, *Pentatrichomonas hominis*, *Enteromonas hominis*, *Balantidium coli*, *Blastocystis hominis*, *Isospora belli*, *Sarcocystis* species, *Cryptosporidium parvum*, *Enterocytozoon bienersi*, *Toxoplasma gondii*, *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium* species, *Babesia microti*, *B. equi*, *B. bigemina*, *Trypanosoma b. gambiense*, *T. b. rhodesiense*, *T. cruzi*, *T. rangeli*, *Leishmania* species and *Pneumocystis carinii*.

#### Brief Summary Text (55):

Evidence that the above-described organisms can be genetically modified using routine methods can be derived from the numerous publications describing protocols, reagents, and other experimental parameters useful therefor. (For example, see 1993 *Protocols in Molecular Parasitology*, ed. J. E. Hyde; Humana Press, Inc., New Jersey; and 1995 *Molecular Approaches to Parasitology*, eds. J. C. Boothroyd & R. Komuniecki; John Wiley & Sons, Inc., New York, the disclosures of both of which are herein incorporated by reference.) Some of the genera routinely studied using art-recognized genetic and molecular techniques include, but are not confined to, *Trypanosoma*, *Leishmania*, *Plasmodium*, *Schistosoma*, *Giardia*, *Theileria*, and *Toxoplasma*. In view of the widely publicized materials and methods for genetically modifying organisms such as those described above, it will be appreciated that merely routine experimentation and routine skill is required to practice this present invention with a particular haploid/diploid unicellular organism, including protozoan and/or parasitic organisms.

#### Detailed Description Text (6):

DNA manipulations. *Leishmania* genomic DNA was prepared by the Triton-LiCl miniprep method and used for Southern blot analysis as described in Ellenberger, T. E., and S. M. Beverley (1989) *J. Biol. Chem.* 264:15094-15103, incorporated herein by reference. Cell slot blots were made as follows. Aliquots (0.1 ml) of stationary-phase cultures were applied to nylon membranes (GeneScreen Plus), prewetted with 2.times.SSPE (1.times.SSPE is 0.15 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>.sub.2, and 1 mM EDTA), with a slot blotter apparatus (Schleicher & Schuell). The cells were lysed by treatment in 0.4 N NaOH-1.5 M NaCl for 10 min and then neutralized in 0.5 M Tris HCl (pH 7.5)-3 M NaCl for 10 min. The DNAs were immobilized by baking, and hybridization was carried out as for the Southern blots. Final washes were performed at 67.degree. C. in 0.1.times.SSPE-0.5% sodium dodecyl sulfate. The DHFR-TS coding-region probe fragment was generated by PCR with genomic DNA as a template. The HYG gene probe was a 1.05-kb BamHI-SpeI fragment from pX63HYG (Cruz, A., C. M. Coburn, and S. M. Beverley (1991) *Proc. Natl. Acad. Sci. USA* 88:7170-7174). The DHFR-TS locus "near flanking" probe was a 2.0-kb EcoRI fragment from pK300 (Kapler, G. M., C. M. Coburn, and S. M. Beverley (1990) *Mol. Cell Biol.* 10:1084-1094, the disclosure of which is herein incorporated by reference), and the "far flanking" probe was a 5.7-kb KpnI fragment from plasmid pLTS-D4AJ11-K57. The *Leishmania* actin probe was a 1.7-kb SalI fragment derived from genomic DNA subcloned in pBluescript. All DNA probes were labeled with [ $\alpha$ .sup.32 P]dCTP by the random-primer method (Felsberg, A. P., and B. Vogelstein (1983) *Anal. Biochem.* 132:6-13).

#### Detailed Description Text (9):

dhfr-ts.sup.- *L. major* E10-5A3 was derived from *L. major* CC-1 by two rounds of targeted gene replacement and bears integrated G418 and hygromycin drug-resistance markers replacing the two DHFR-TS alleles. CC-1 is a clone of strain LT252 (MHOM/IR/83/LT252; Kapler et al. (1990), 10 *Mol. Cell Biol.* 10:1084-1094). Virulent challenge *L. major* was clone 5 of the LV39 line (Rho/SU/59/P; Marchand et al. (1987), 9 *Parasite Immunol.* 81-92); it was passed through mice every 4 weeks to maintain virulence. When propagated for immunization or challenges, *Leishmania* were grown on biphasic NNN medium (Titus et al. (1991), 21 *Eur. J. Immunol.* 559-567; thymidine at 10 .mu.g/ml was added for



dhfr-ts.sup.-). Metacyclic dhfr-ts.sup.- promastigotes were purified by using peanut agglutinin (Sacks, D. L. & Perkins, P. V. (1984) Science 223, 1417-1419) and a freeze-thaw lysate was made (Titus, R. G., Muller, I., Kimsey, P., Cerny, A., Behin, R. Zinkernagel, R. & Louis, J. (1991) Eur. J. Immunol. 21, 559-567). BALB/c and BALB/c athymic nu/nu mice were obtained from the National Cancer Institute (Frederick, Md.); CBA/T6 mice were obtained from The Jackson Laboratory. Subcutaneous injections (s.c.) were delivered at a shallow site in the hind footpad, i.v. inoculations were into the tail vein, and i.m. injections were into the large rear leg muscle mass. Lesion progression was followed by using a vernier caliper (Titus, R. G., Muller, I., Kimsey, P., Cerny, A., Behin, R. Zinkernagel, R. & Louis, J. (1991) Eur. J. Immunol. 21, 559-567). Macrophages were elicited with starch in the peritoneum of BALB/c mice, harvested, and infected with parasites, and their parasite burden was assessed as described (Titus, R. G., Kelso, A., & Louis, J. A. (1984) Clin. Exp. Immunol. 55, 157-165). The limiting dilution assay for enumerating parasites in infected mouse tissues was performed as described (Titus, R. G., Marchand, M., Boon, T. & Louis, J. A. (1985) Parasite Immunol. 7, 545-555). Lymph node cell proliferation assays were performed as described (Kimsey, P. B., Theodos, C. M., Mitchen, T. K., Turco, S. J. Titus, R. G. (1993) Infect. Immun. 61, 5205-5213). Osmotic pumps (14-day Alzet model 2002; Alza) were loaded with thymidine at 42 mg/ml and implanted 1 day prior to infection with dhfr-ts.sup.-.

Detailed Description Text (14):

A. A protocol for null targeting of diploid cells was developed, in which transfection of a DHFR-TS deletion construct into *Leishmania* cells followed by negative selection yielded parasites lacking exemplary DHFR-TS or foreign sequences.

Detailed Description Text (16):

Selection against DHFR-TS in *L. major*. In prokaryotes, loss of TS activity confers transient resistance to antifolates if thymidine is provided (Bertino, J. B. and K. A. Stacey (1966) Biochem J. 101:32C-33C; Stacey, K. A. and E. Simson (1965) J. Bacteriol. 90:554-555). This stems from a sparing of reduced-folate pools, since TS is the only significant enzyme whose activity oxidizes tetrahydrofolates. It was predicted that the absence of the bifunctional DHFR-TS would confer a similar if not stronger phenotype in *Leishmania* spp. as a result of linkage of the two enzymatic activities. This was tested with a panel of cells, obtained by homologous gene replacement, which contained either one (+/HYG) or no (NEO/HYG) copies of DHFR-TS. In liquid media, wild-type or heterozygous cells show typical sensitivities to MTX (EC<sub>50</sub> about 1  $\mu$ M), while the dhfr-ts.sup.- NEO/HYG knockout grew well at 100  $\mu$ M MTX. Permanent MTX resistance may arise from the provision of reduced folates by the alternate pteridine reductase PTR1 in *L. major*.

Detailed Description Text (17):

These data suggested that MTX+Tdr could be used as a selection against *Leishmania* DHFR-TS. Accordingly, 20.times.10.sup.6 +/-HYG *Leishmania* cells were plated on medium containing MTX-Tdr, and colonies appeared at a frequency of 0.7.times.10.sup.-5 to 2.5.times.10.sup.-5 per cell plate. In contrast, wild-type cells bearing two copies of DHFR-TS (+/+) did not give rise to any colonies. Omission of Tdr also gave no colonies, suggesting that those obtained with Tdr did not arise from common MTX resistance mechanisms, such as amplification of DHFR-TS or PTR1, mutations in DHFR, or from altered MTX accumulation, as described previously for numerous *Leishmania* lines and species (Arrebola, R., A. Olmo, P. Reche, E. P. Garvey, D. V. Santi, L. M. Rulz-Perez, and D. Gonzalez-Pacanowska (1994) J. Biol. Chem. 269:10590-10596; Beverley, S. M. (1991) Annu. Rev. Micro-biol. 45:417-466; Borst, P. and M. Ouellette (1995) Annu. Rev. Microbiol. 49:427460; Ellenberger, T. E. and S. M. Beverley (1987) J. Biol. Chem. 262:13501-13506).

Detailed Description Text (20):

Null-targeting strategies as a general tool. Using the negative DHFR-TS selection and transfection of a null-targeting fragment, it was possible in one step to generate *Leishmania* spp. deleted for both copies of DHFR-TS. This null-targeting approach could be applied at any locus for which an appropriate negative selection can be devised, without sacrificing any selectable marker. A large number of loci are potentially suitable targets for this purpose in different species, including TK, the orotidine 5'-phosphate-decarboxylase locus, HGPRT, APRT, TS, or DHFR-TS. This protocol could also be used to introduce any given coding region directly into the genome, by construction of a null targeting fragment in which the negative selectable gene (DHFR-TS here) was replaced with another coding region. This would be advantageous in many circumstances and these approaches can be applied to cells from any diploid species. The null-targeting methods disclosed herein can also be used to delete a single allele or

copy of a given genetic locus in a diploid, by applying appropriate intermediate levels of negative selection to select against only one of the alleles or genetic loci. The resulting colonies can be screened for the presence of both a wild-type copy and a deleted copy of the allele or genetic locus, using Southern blot analysis methods known in the art.

Detailed Description Text (21):

B. Selection against DHFR-TS on a heterozygous line (+/HYG) was used to generate colonies exhibiting both loss of heterozygosity (LOH) and structural mutations in DHFR-TS, permitting the first measurement of mutation frequencies in this parasite. Loss of heterozygosity occurred at a frequency ranging from  $10^{-4}$  to  $10^{-6}$  and was elevated 24-fold by treatment with  $\gamma$ -irradiation, while the frequency of other events was less than  $10^{-6}$  and was increased more than 1,000-fold by nitroguanidine treatment. The frequency of loss of heterozygosity relative to other processes such as mutation and gene replacement has important implications for genetic variability in natural *Leishmania* populations and the generation of both targeted and random mutations.

Detailed Description Text (24):

The HYG copy number in several DTS+ and DTS.DELTA. lines was measured in Southern blot and slot blot analysis, using hybridization with a *Leishmania* actin probe to control for DNA loading. Assuming the HYG copy number in the parent +/HYG to be 1, values approaching 2 were found for the DTS.DELTA. lines ( $1.7 \pm 0.2$ ,  $1.7 \pm 0.3$ ,  $1.6 \pm 0.3$ , and  $1.5 \pm 0.2$  [n=4] for colonies LH1, LOH 5-1, LOH 5-2, and LOH 5-3, respectively) and close to 1 for the DTS+ lines ( $0.7 \pm 0.2$  and  $0.8 \pm 0.2$  for colonies F22 and PM13, respectively). In combination with the Southern blot data described above, it appeared that the LOH event in the DTS.DELTA. lines did not occur by chromosome loss but arose from an event where loss of the DHFR-TS allele was coupled to duplication of the HYG allele. Potential mechanisms include gene conversion; mitotic crossing over, or some form of chromosome mis-segregation.

Detailed Description Text (26):

The rate of increase of colony formation on MTX+TdR plates was measured as a function of the period of growth at higher hygromycin B concentrations. On average, every three passages resulted in about a 10-fold increase in colony formation. Again, no correlation was found between hygromycin B concentration and the frequency of colonies on MTX+TdR plates. The DHFR-TS phenotype in the colonies arising from these studies were not systematically examined, but those tested always belonged to the DTS.DELTA. class. Thus, elevated drug pressure can be used effectively to induce homozygosity from heterozygous replacements in *Leishmania* major.

Detailed Description Text (27):

The system established here has several direct applications. First, the defective DHFR-TS mutants can be used to explore the role of particular amino acids in the activity or stability of this key metabolic protein. Second, the type and frequency of mutations induced by other mutagenic treatments can be characterized, and these data incorporated into improved mutant recovery protocols as outlined above. It would be particularly interesting to examine the effects of oxidative stress on mutagenesis, since the entry of *Leishmania* cells into, and propagation within, the phagolysosome of the vertebrate macrophage may expose the parasite to this class of DNA-damaging agents as part of the normal infectious cycle. Potentially, measurement of alterations at DHFR-TS within the DHFR-TS/HYG heterozygote can be used as a probe of the exposure of *Leishmania* spp. to mutagenic stress during the natural infectious cycle.

Detailed Description Text (28):

The occurrence of LOH has important uses in the conservation of genetic markers in transfectional manipulations of the *Leishmania* genome. The selection of +/HYG DHFR-TS heterozygote with elevated hygromycin B levels rapidly gave rise to thy<sup>sup</sup>- parasites completely lacking DHFR-TS. This permits the use of a single targeting construct and transfection to obtain null mutants for genes for which negative selections are unavailable. Of course, the LOH-based approach can be accelerated by the application of negative selections. Homozygous HYG/HYG replacements at the LPG2 locus of *L. donovani* by lectin selection against lipophosphoglycan expression were generated in this manner (Descoteaux, A. H., et al., (1995) Science 269:1869-1873, incorporated herein by reference).

Detailed Description Text (36):

To test whether mariner could transpose in *Leishmania*, two plasmids were constructed: pX63TKNEO-TPASE, containing the transposase gene under the control of leishmanial

sequences necessary for its expression, and pX63PAC-Mos1, containing an intact copy of the transposon. The plasmids were sequentially introduced into the L. major line +/-DELTA.1, and 22 independent double transfectants were analyzed for mariner transpositions by Southern blot hybridization. Five out of 22 clones showed extra mariner hybridizing bands distinct from the ones corresponding to the input plasmids suggesting the occurrence of transposition. Several of these bands were isolated by inverse PCR, and analysis of their sequences demonstrated that they had the structure expected for true transpositions, with an intact transposon end followed by a TA dinucleotide and a stretch of DNA not present in the donor plasmids. Southern blot experiments using inverse PCR products as probes identified bands in the parental +/-DELTA.1 line that were increased by about 1.3 kb, the expected for a mariner insertion, in the clone that gave rise to the PCR product, providing further evidence that they represent "bona-fide" transposition events and not PCR artifacts. Assuming that all the novel mariner bands detected in this experiment are due to transposition, we conclude that mariner is being mobilized at a very high frequency, in about 20% of the colonies tested.

#### Detailed Description Text (37):

Tests were performed to determine whether heterologous expression of mariner could produce the insertional inactivation of a *Leishmania* gene. The DHFR-TS locus was chosen as the target, as it is hemizygous in +/-DELTA.1 and because we can select for its inactivation. Plating of mariner-containing strains on methotrexate (MTX) and thymidine produced dhfr-ts.sup.- mutants at an overall frequency of  $1.2 \times 10^{-4}$  and with a distribution of loss of heterozygosity and mutational events. Among the dhfr-ts.sup.- mutants that retained DHFR-TS sequences, one clone, 22M3, showed a larger DHFR-TS allele in Southern blots. The increase in size (about 1.3 kb) suggested the insertion of mariner in DHFR-TS and, indeed, rehybridization of the blot with a mariner probe-identified a band in 22M3 identical in size to the DHFR-TS band. Sequencing of the insertion in 22M3 confirmed that mariner transposed into DHFR-TS, more precisely, into a TA dinucleotide located at position 532 of the coding region. Interestingly, the insertion of mariner at DHFR-TS led to duplication of the target TA. This result, together with the sequences of the transpositions, which always show a TA flanking the 5' and 3' end of the transposon, suggest that mariner transposition in *Leishmania* leads to target site duplication and thus occurs by the typical mechanism described for the mariner/Tc1 family. Considering that disruption of DHFR-TS by mariner represented 1/48 of the dhfr-ts.sup.- lines analyzed, we can derive a frequency of  $2.5 \times 10^{-6}$  for the insertional inactivation of one allele of a gene in *Leishmania*. This frequency becomes about  $10^{-1}$  /genome, if we take into account that DHFR-TS represents about 1/30,000 of the parasite's nuclear DNA.

#### Detailed Description Text (39):

Next, it was determined whether a modified mariner element carrying a drug resistance marker could be used in a "gain of function" selection for mRNA processing signals in the *Leishmania* genome. The selectable mariner, named MosHYG, has a hygromycin resistance gene (HYG) inserted next to its 5' end and is cloned in the vector pX63PAC in the anti-sense orientation relative to the *Leishmania* trans-splicing signals, such that no processed HYG message can be produced. MosHYG has in-frame stop codons upstream of the HYG ATG and therefore should yield only transcriptional fusions. Transfections with pX63PAC-MosHYG produced hundreds of colonies when plated on puromycin and no colonies when plated on hygromycin confirming that HYG is silent in the context of the donor plasmid. However, transposase-expressing lines carrying pX63PAC-MosHYG gave rise to hygromycin-resistant (hyg.sup.r) colonies at a frequency ranging from less than  $10^{-6}$  to  $10^{-3}$  when replated in 32  $\mu$ g/ml of the drug, suggesting that the element was moving. Activation of HYG due to transposition should produce chimeric MosHYG mRNA's: containing *Leishmania* sequences preceded by the ubiquitous trypanosomatid spliced leader on the 5' end. We tested this using the well-known RT-PCR and found that MosHYG had indeed transposed to several different *Leishmania* loci, most of which didn't show any matches in sequence data bases. One of the cDNAs (T1.2D) identified an event that trapped the splice acceptor site and 5' untranslated region of DHFR-TS. This corresponds to a transposition within the donor plasmids which use DHFR-TS flanking sequences to drive the drug markers, and demonstrated that our selection for processing signals was working as expected. Northern analysis using probes derived from the uncharacterized loci identified transcripts in wild type *Leishmania* that are increased in size the hyg.sup.r clone that originated the RT-PCR product. This confirmed that the chimeric mRNAs are not PCR artifacts and that an engineered mariner can generate gene fusions capable of identifying *Leishmania* genetic elements.

#### Detailed Description Text (40):

Additionally, it is understood that the methods described herein can be used to identify genetic elements conferring different levels of expression or under different types of regulatory control. A modified mariner element analogous to MosHYG, but wherein the hygromycin gene is replaced by the Green Fluorescent Protein gene (Ha, Schwartz, Turco, Beverley, Molec. Biochem. Parasitol. 77: 57-64, 1996, incorporated herein by reference), can be used to identify these different types of genetic elements. Transposition events are generated using the methods described above. Transposition events that result in Green Fluorescent Protein expression (productive transposition events) can be identified by assaying Green Fluorescent Protein expression using methods known in the art and described in Example 6. Expression of Green Fluorescent Protein in *Leishmania* can be assayed in culture medium, in cultured macrophages and in vivo. Different productive transposition events will give rise to different levels of Green Fluorescent Protein expression, and to expression under different growth conditions. The genetic elements responsible for these different levels and patterns of expression can be readily identified, using methods known in the art, by studying the sequences flanking the site of insertion of the modified transposon. It is also expected that genetic elements can be identified in other unicellular organisms using the methods described herein, in combination with methods known in the art, and only routine experimentation.

Detailed Description Text (44):

A. Vaccination with live *Leishmania* major has been shown to yield effective immunization in humans; however, this has been discontinued because of the problems associated with virulence of the available vaccine line. To circumvent this, the ability of a dhfr-ts.sup.- null mutant (E10-5A3) of *L. major*, obtained by gene targeting, to infect and then to vaccinate mice against challenge with virulent *L. major* was tested. Survival and replication of dhfr-ts.sup.- in macrophages in vitro were dependent upon thymidine, with parasites differentiating into amastigotes prior to destruction. dhfr-ts.sup.- parasites persisted in BALB/c mice for up to 2 months, declining with a half-life of 2-3 days. Nonetheless, dhfr-ts.sup.- was incapable of causing disease in both susceptible and immunodeficient (nu/nu) BALB/c mice. Animal infectivity could be partially restored by thymidine supplementation. When inoculated by the i.v., s.c., or i.m. routes into mice, dhfr-ts.sup.- could elicit substantial resistance to a subsequent challenge with virulent *L. major*. Thus, *Leishmania* bearing auxotrophic gene knock-outs can be safe and induce protective immunity. As described earlier, such a genetically modified also can be used as a platform for delivery of immunogens relevant to other diseases.

Detailed Description Text (48):

With BALB/c mice, vaccination with purified metacyclic dhfr-ts.sup.- was as effective as stationary-phase dhfr-ts.sup.-. A metacyclic dhfr-ts.sup.- lysate did not confer protection, other workers have also reported that parasite lysates are ineffective. Similar results were obtained when mice were challenged 1 month after vaccination. Vaccination by the s.c. route imparted minimal protection.

Detailed Description Text (54):

Despite the block to propagation and pathogenesis, the low thymidine levels available to *Leishmania* in vivo are apparently sufficient to prevent or delay classic rapid thymine-less death. This follows because dhfr-ts.sup.- did not perish immediately in vivo but, instead, slowly declined over a period of months. Complete removal of thymidine results in rapid death within a few days in vitro (Cruz, A. & Beverley, S. M. (1990) Nature (London) 348, 171-174, the disclosure of which is herein incorporated by reference). Thus, subtle interactions between dhfr-ts.sup.- and the host exist that promote limited persistence simultaneously with differentiation. Minimally, this should prolong the period of exposure to both live and dead parasite antigens, while differentiation of dhfr-ts.sup.- would deliver substantial quantities of amastigote antigens. These features are advantageous to vaccination efforts and may perhaps be unique to dhfr-ts.sup.- knockouts, relative to other potential candidate attenuating loci that we have considered.

Detailed Description Text (55):

B. Marker-free dhfr-ts.sup.- *Leishmania* knockouts as attenuated live vaccine line. In many situations, the introduction of selectable markers may not always be desirable, as in organisms destined for certain uses outside the laboratory. For example, it was shown previously that dhfr-ts.sup.- parasites containing marker genes have potential as live, attenuated vaccines against cutaneous leishmaniasis in a susceptible mouse model (Titus, R. G., F. J. Guelros-Filbo, L. A. R. DeFreitas, and S. M. Beverley (1995) Proc. Natl. Acad. Sci. USA 92:10267-10271, the disclosure of which is herein incorporated by reference). The NEO resistance marker present in this line can inactivate the

aminoglycoside paromomycin, which shows some efficacy in antileishmanial chemotherapy (Gueiros-Filbo, F. J. and S. M. Beverley (1994) Exp. Parasitol. 78:425-428, the disclosure of which is herein incorporated by reference). It is possible that NEO genes from auxotrophic vaccine lines could find their way into natural field populations, thereby possibly compromising paromomycin therapy. The use of marker-free knockouts as described herein above in Examples 2 and 3 circumvents this problem, because they lack any selectable marker gene coding sequences. Data are available from tests in murine models which indicate that the efficacy of a marker-free mutant as a live vaccine is uncompromised and comparable to that of the previously studied marker-containing (NEO/HYG) dhfr-ts.sup.- null mutant. Moreover, data are available in non-human primate models which indicate that the dhfr-ts.sup.- parasite does not cause any pathology in this system as well.

Detailed Description Text (59):

As a further example, it has been shown (LeBowitz, Coburn, McMahon-Pratt and Beverley, PNAS 87:9736-9740 1990, incorporated herein by reference) that Leishmania promastigotes, transfected with the expression vector pX containing an inserted .beta.-galactosidase gene, contain nearly 1% of their total cellular protein as .beta.-galactosidase. Infective parasites containing this same construct were prepared and .beta.-galactosidase levels determined in promastigotes and amastigotes (infective macrophage stage of Leishmania) recovered from lesions of infected mice. Relative to promastigotes, amastigotes synthesized about 10% as much .beta.-galactosidase, which would correspond to about 0.1% of total cellular protein. Similar experiments carried out with a newer expression vector, pXG (Ha, Schwarz, Turco, Beverley, Molec. Biochem. Parasitol. 77:57-64 1996, incorporated herein by reference) show that relative to promastigotes, amastigotes synthesized about 50% as much .beta.-galactosidase. Similar experiments can be carried out using Green Fluorescent Protein instead of .beta.-galactosidase. Green Fluorescent Protein can be effectively expressed in Leishmania, and its expression can be monitored by fluorescence. These data confirm the ability to create genetically modified parasites synthesizing high levels of a foreign, heterologous protein in the desired parasite stage infecting the host.

Detailed Description Text (63):

Starch-elicited peritoneal macrophages were infected in vitro with virulent Leishmania (LV39 clone 5) or dhfr-ts.sup.-. Both lines were taken up by the macrophages. Beyond 24 hr, virulent Leishmania continued to replicate as amastigotes within the macrophages. In contrast, dhfr-ts.sup.- did not replicate. Significantly, after 24 or 48 hr, dhfr-ts.sup.- within macrophages appeared morphologically as amastigotes, even in the absence of thymidine. To confirm that the disablement of dhfr-ts.sup.- arose specifically from the lack of DHFR-TS, we added thymidine (100 .mu.g/ml) to the medium, which restored both survival and replication.

Detailed Description Text (64):

In several experiments, attempts were made to rescue the dhfr-ts.sup.- phenotype in vivo, by implanting osmotic pumps that delivered thymidine at the maximum tolerated dose for either 14 or 28 days. Small lesions (up to 0.25 mm) were obtained after infection with 10.sup.8 dhfr-ts.sup.-. The small size, relative to infection with CC-1, may be due to (i) the rapid clearance of thymidine the bloodstream, (ii) deliver of insufficient thymidine to the Leishmania phagolysosomal compartment, and/or (iii) a need in vivo but not in vitro for reduced folates, beyond the capacity of the alternative pteridine reductase PTR1. Significantly, upon removal of the thymidine pumps, dhfr-ts.sup.- lesions regressed immediately and disappeared within 2 weeks.

Detailed Description Text (66):

The studies above addressed infectivity by the criterion of visible lesion development. Since Leishmania can persist in the absence of overt disease, the number of viable dhfr-ts.sup.- parasites was measured after infection. Susceptible BALB/c mice were injected s.c. in one hind footpad with 10.sup.8 dhfr-ts.sup.-. Parasites and, at various intervals, were sacrificed, and parasites were enumerated in the footpad and the draining lymph node. The dhfr-ts.sup.- parasites persisted for approximately 2 months, although their numbers declined with a half-life of 2-3 days. From the macrophage results, we infer that the persisting parasites were amastigotes.

Detailed Description Text (78):

A particularly preferred delivery device for this indication is a species of Leishmania expressing glucocerebrosidase enzyme, Ceredase.RTM., Cerezyme.RTM., or equivalents and/or variants of the foregoing, because Leishmania can reside in the macrophages of a diseased individual, the actual site of the primary disease-producing event. It is not required, however, that the present device reside in macrophages; it is known that

lysosomal enzymes can be transferred by endocytosis or by cell-to-cell transfer (see 1996 Goodman & Gilman's The Pharmaceutical Basis of Therapeutics, pp. 1487-1518; eds. J. G. Hardman, L. E. Limbird, P. B. Molinoff, R. W. Ruddon, & A. Goodman Gilman, 9th ed., McGraw-Hill, N.Y.). Additionally, it is anticipated that the present device can express glucocerebrosidase enzyme, Ceredase.RTM., Cerezyme.RTM., or equivalents and/or variants of the foregoing, such that it is secreted. Alternatively, it can be associated with the device, for example the outer surface of the device to name but one alternative. All that is required is that the device provide enzyme to the diseased individual.

Detailed Description Text (87):

With respect to routes of delivery, insulin can be administered orally, nasally, rectally and by subcutaneous means such as injections or implantation. The objective is to identify a route which provides higher relative concentrations of insulin in the portal circulation. Again, the present device is particularly useful because it can be designed to reside in a particular tissue, especially one which would most effectively deliver insulin to the portal circulation. A currently preferred tissue is skin. As explained earlier, *Leishmania* spp., particularly less virulent variants, can reside in the skin of the host, thereby permitting the skilled practitioner to target the present device to skin, if desired.

Detailed Description Text (104):

Where the device comprises part of a tissue or organ preservation solution, any commercially available preservation solution can be used to advantage. For example, useful solutions known in the art include Collins solution, Wisconsin solution, Belzer solution, Eurocollins solution and lactated Ringer's solution. Generally, an organ preservation solution usually possesses one or more of the following properties: (a) an osmotic pressure substantially equal to that of the inside of a mammalian cell (solutions typically are hyperosmolar and have K<sup>+</sup> and/or Mg<sup>++</sup> ions present in an amount sufficient to produce an osmotic pressure slightly higher than the inside of a mammalian cell); (b) the solution typically is capable of maintaining substantially normal ATP levels in the cells; and (c) the solution usually allows optimum maintenance of glucose metabolism in the cells. Organ preservation solutions also can contain anticoagulants, energy sources such as glucose, fructose and other sugars, metabolites, heavy metal chelators, glycerol and other materials of high viscosity to enhance survival at low temperatures, free oxygen radical inhibiting and/or scavenging agents and a pH indicator. A detailed description of preservation solutions and useful components can be found, for example, in U.S. Pat. No. 5,002,965, the disclosure of which is incorporated herein by reference.

Current US Cross Reference Classification (1):

424/269.1

Other Reference Publication (3):

Avraham Laban et al., (1989) "Transfection of *Leishmania enrietti* and expression of chloramphenicol acetyltransferase gene" vol. 86, Proc. Natl. Acad. Sci. USA, pp. 9119-9123.

Other Reference Publication (5):

Geoffrey M. Kapler et al., (1990) "Stable Transfection of the Human Parasite *Leishmania* major Delineates a 30-Kilobase Region Sufficient for Extrachromosomal Replication and Expression" vol. 10, Molecular and Cellular Biology, pp. 1084-1094.

Other Reference Publication (6):

Avraham Laban et al., (1990) "Stable expression of the bacterial neo' gene in *Leishmania enriettii*" vol. 343, Nature, pp. 572-574.

Other Reference Publication (9):

Jonathan H. LeBowitz et al. (1990) "Development of a stable *Leishmania* expression vector and application to the study of parasite surface antigen genes" vol. 87, Proc. Natl. Acad. Sci. USA, pp. 9736-9740.

Other Reference Publication (11):

James F. Tobin et al., (1991) "Homologous recombination in *Leishmania enriettii*" vol. 88, Proc. Natl. Acad. Sci. USA, pp. 864-868.

Other Reference Publication (14):

James F. Tobin et al., (1992) "A Sequence Insertion Targeting Vector for *Leishmania enriettii*" vol. 267, The Journal of Biological Chemistry, pp. 4752-4758.

Other Reference Publication (15):

James F. Tobin et al., (1993) "Mutational analysis of a signal sequence required for protein secretion in Leishmania major" vol. 62, Molecular and Biochemical Parasitology, pp. 243-250.

Other Reference Publication (18):

Daniel J. Freedman et al., (1993) "Two more independent selectable markers for stable transfection of Leishmania" vol. 62, Molecular and Biochemical Parasitology, pp. 37-44.

Other Reference Publication (19):

James F. Tobin et al., (1993) "Transfected Leishmania Expressing Biologically Active IFN- $\gamma$ " vol. 150, The Journal of Immunology, pp. 5059-5069.

Other Reference Publication (21):

Barbara Papadopoulou et al., (1994) "Changes in Folate and Pterin Metabolism after Disruption of the Leishmania H Locus Short Chain Dehydrogenase Gene" vol. 269, The Journal of Biological Chemistry, pp. 7310-7315.

Other Reference Publication (22):

Frederico J. Gueiros-Filho et al., (1994) "On the introduction of Genetically Modified Leishmania outside the Laboratory" vol. 78, Experimental Parasitology, pp. 425-428.

Other Reference Publication (23):

Richard G. Titus et al., (1995) "Development of a safe live Leishmania vaccine line by gene replacement" vol. 92, Proc. Natl. Acad. Sci. USA, pp. 10267-10271.

Other Reference Publication (25):

Wen Wei Zhang et al., (1995) "The expression of biologically active human p53 in Leishmania cells: a novel eukaryotic system to produce recombinant proteins", vol. 23, Nucleic Acids Research, pp. 4073-4080.

Other Reference Publication (29):

Larry M. Chow et al., "Cloning and functional analysis of an extrachromosomally amplified multidrug resistance-like gene in Leishmania enriettii" vol. 60, Molecular and Biochemical Parasitology, pp. 195-208 (1993).

Other Reference Publication (31):

Albert Descoteaux et al., "A Specialized Pathway Affecting Virulence Glycoconjugates of Leishmania" vol. 269, Science, pp. 1869-1872 (1995).

Other Reference Publication (35):

LeBowitz et al., "Development of a stable Leishmania expression vector and application to the study of parasite surface antigen genes" vol. 87, Proc. Natl. Acad. Sci. USA, pp. 9736-9740 (Dec. 1990).

Other Reference Publication (36):

Richard G. Titus et al., (1995) "Development of a safe live Leishmania vaccine line by gene replacement" vol. 92, Proc. Natl. Acad. Sci. USA, pp. 10267-10271.

Other Reference Publication (37):

Gueiros-Filho et al., "Construction of a Leishmania major DHFR-TS knock-out without selectable markers and its use for live vaccination", Molecular Parasitology Meeting, (Sep. 17, 1995).

Other Reference Publication (38):

Gueiros-Filho et al., "'In vivo' transposition in Leishmania", Molecular Parasitology Meeting, (Sep. 15, 1996).

Other Reference Publication (39):

Gueiros-Filho et al., "Selection against the Dihydrofolate Reductase-Thymidylate Synthase (DHFR-TS) Locus as a Probe of Genetic Alterations in Leishmania major", Molecular and Cellular Biology (Oct. 1996, p. 5655-5663).

Other Reference Publication (40):

Curotto de Lafaille et al., "Creation of Null/+Mutants of the  $\alpha$ -Tubulin Gene in Leishmania enrietti by Gene Cluster Deletion", The Journal of Biological Chemistry, (Nov. 25, 1992 p. 23839-23846).

Other Reference Publication (43):

Hwang, et al. "Creation of Homozygous Mutants of Leishmania donovani with Single Targeting Constructs", The Journal of Biological Chemistry, (Nov. 29, 1996, p. 30840-30846).

## CLAIMS:

1. A method for producing an expression product in Leishmania, the method comprising the steps of:

transfecting a Leishmania organism with a nucleic acid comprising a nucleotide sequence encoding an expression product and a nucleotide sequence complementary to a wild type nucleotide sequence flanking a genetic locus in the genome of said organism; and,

selecting for a conditionally defective phenotype generated by loss of said genetic locus from said genome,

wherein the introduction of said nucleic acid into said organism results in the production of said expression product.

2. A method for producing an expression product from a Leishmania organism, the method comprising the steps of:

providing a conditionally defective Leishmania organism that expresses an expression product, wherein said conditionally defective organism was produced by selecting for a conditionally defective phenotype generated by loss of a genetic locus from the genome of a Leishmania organism transfected with a nucleic acid comprising a nucleotide sequence encoding said expression product and a nucleotide sequence complementary to a wild type nucleotide sequence flanking said genetic locus; and,

growing said conditionally defective organism to produce said expression product.



[Federal Register: August 5, 2002 (Volume 67, Number 150)]

[Notices]

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[DOCID:fr05au02-40]

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DEPARTMENT OF DEFENSE

Department of the Army

Availability for Non-Exclusive, Exclusive, or Partially Exclusive  
Licensing of U.S. Patent Application Concerning Microfluidized  
Leishmania Lysate and Methods of Making and Using Thereof

AGENCY: Department of the Army, DOD.

ACTION: Notice.

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SUMMARY: In accordance with 37 CFR 404.6 and 404.7, announcement is  
made of the availability for licensing of U.S. Patent Application No.  
09/975,020 entitled "Microfluidized Leishmania Lysate and Methods of  
Making and Using Thereof," **filed** October 12, 2001. Foreign rights  
(PCT/US01/31894) are also available. The United States Government, as  
represented by the Secretary of the Army has rights in this invention.

ADDRESSES: Commander, U.S. Army Medical Research and Materiel Command,  
ATTN: Command Judge Advocate, MCMR-JA, 504 Scott Street, Fort Detrick,  
Frederick, MD 21702-5012.

FOR FURTHER INFORMATION CONTACT: For patent issues, Ms. Elizabeth  
Arwine, Patent Attorney, (301) 619-7808. For licensing issues, Dr. Paul  
Mele, Office of Research & Technology Assessment, (301) 619-6664, both  
at telefax (301) 619-5034.

SUPPLEMENTARY INFORMATION: This invention relates generally to  
microfluidized Leishmania lysate preparations. In particular, the  
present invention relates to microfluidized Leishmania lysate  
preparations for assays and immunogenic compositions.

Luz D. Ortiz,  
Army Federal Register Liaison Officer.  
[FR Doc. 02-19715 Filed 8-2-02; 8:45 am]  
BILLING CODE 3710-08-M

**ACTION:** Notice.

**SUMMARY:** In accordance with 37 CFR 404.6 and 404.7, announcement is made of the availability for licensing of U.S. Patent Application No. 09/949,572 entitled "Free Floating Cryostat Sections for Immunolectron Microscopy," filed September 10, 2001. Foreign rights are also available (PCT/US01/28340). The United States Government, as represented by the Secretary of the Army has rights in this invention.

**ADDRESSES:** Commander, U.S. Army Medical Research and Materiel Command, ATTN: Command Judge Advocate, MCMR-JA, 504 Scott Street, Fort Detrick, Frederick, MD 21702-5012.

**FOR FURTHER INFORMATION CONTACT:** For patent issues, Ms. Elizabeth Arwine, Patent Attorney, (301) 619-7808. For licensing issues, Dr. Paul Mele, Office of Research & Technology Assessment, (301) 619-6664, both at telefax (301) 619-5034.

**SUPPLEMENTARY INFORMATION:** This invention relates to the field of histology and immuno-histology using immunolectron microscopy. More specifically, this invention relates to the field of free-floating cryostat sections for use in light and electron microscopy to bridge the gap between these two viewing mediums.

Luz D. Ortiz,  
Army Federal Register Liaison Officer.  
[FR Doc. 02-19709 Filed 8-2-02; 8:45 am]  
BILLING CODE 3710-08-M

**DEPARTMENT OF DEFENSE****Department of the Army**

**Availability for Non-Exclusive, Exclusive, or Partially Exclusive Licensing of U.S. Patent Application Concerning Heterologous Protection Induced by Immunization With Invaplex Vaccine**

**AGENCY:** Department of the Army, DoD.  
**ACTION:** Notice.

**SUMMARY:** In accordance with 37 CFR 404.6 and 404.7, announcement is made of the availability for licensing of U.S. Patent Application No. 10/150,814 entitled "Heterologous Protection Induced by Immunization with Invaplex Vaccine," filed May 17, 2002. Foreign rights (PCT/US02/16029) are also available. The United States Government, as represented by the Secretary of the Army has rights in this invention.

**ADDRESSES:** Commander, U.S. Army Medical Research and Materiel Command, ATTN: Command Judge Advocate, MCMR-JA, 504 Scott Street, Fort Detrick, Frederick, Maryland 21702-5012.

**FOR FURTHER INFORMATION CONTACT:** For patent issues, Ms. Elizabeth Arwine, Patent Attorney, (301) 619-7808. For licensing issues, Dr. Paul Mele, Office of Research & Technology Assessment, (301) 619-6664, both at telefax (301) 619-5034.

**SUPPLEMENTARY INFORMATION:** In this application is described a composition, Invaplex, derived from a gram negative bacteria for use in generating an immune response in a subject against one or more heterologous species or strains of gram-negative bacteria.

Luz D. Ortiz,  
Army Federal Register Liaison Officer.  
[FR Doc. 02-19710 Filed 8-2-02; 8:45 am]  
BILLING CODE 3710-08-M

**DEPARTMENT OF DEFENSE****Department of the Army**

**Availability for Non-Exclusive, Exclusive, or Partially Exclusive Licensing of U.S. Patent Application Concerning Microfluidized Leishmania Lysate and Methods of Making and Using Thereof**

**AGENCY:** Department of the Army, DOD.  
**ACTION:** Notice.

**SUMMARY:** In accordance with 37 CFR 404.6 and 404.7, announcement is made of the availability for licensing of U.S. Patent Application No. 09/975,020 entitled "Microfluidized Leishmania Lysate and Methods of Making and Using Thereof," filed October 12, 2001. Foreign rights (PCT/US01/31894) are also available. The United States Government, as represented by the Secretary of the Army has rights in this invention.

**ADDRESSES:** Commander, U.S. Army Medical Research and Materiel Command, ATTN: Command Judge Advocate, MCMR-JA, 504 Scott Street, Fort Detrick, Frederick, MD 21702-5012.

**FOR FURTHER INFORMATION CONTACT:** For patent issues, Ms. Elizabeth Arwine, Patent Attorney, (301) 619-7808. For licensing issues, Dr. Paul Mele, Office of Research & Technology Assessment, (301) 619-6664, both at telefax (301) 619-5034.

**SUPPLEMENTARY INFORMATION:** This invention relates generally to microfluidized *Leishmania* lysate

preparations. In particular, the present invention relates to microfluidized *Leishmania* lysate preparations for assays and immunogenic compositions.

Luz D. Ortiz,  
Army Federal Register Liaison Officer.  
[FR Doc. 02-19715 Filed 8-2-02; 8:45 am]  
BILLING CODE 3710-08-M

**DEPARTMENT OF DEFENSE****Department of the Army**

**Availability for Non-Exclusive, Exclusive, or Partially Exclusive Licensing of U.S. Patent Applications Concerning Specific Inhibitors and Therapeutic Agents for Botulinum Toxin B and Tetanus Neurotoxins**

**AGENCY:** Department of the Army, DOD.  
**ACTION:** Notice.

**SUMMARY:** In accordance with 37 CFR 404.6 and 404.7, announcement is made of the availability for licensing of the related U.S. patent applications concerning "Specific Inhibitors and therapeutic Agents for Botulinum Toxin B and Tetanus Neurotoxins" listed below. The United States Government, as represented by the Secretary of the Army, has rights in these inventions. Foreign rights are also available.

**ADDRESSES:** Commander, U.S. Army Medical Research and Materiel Command, ATTN: Command Judge Advocate, MCMR-JA, 504 Scott Street, Fort Detrick, Frederick, MD 1702-5012.

**FOR FURTHER INFORMATION CONTACT:** For patent issues, Ms. Elizabeth Arwine, Patent Attorney, (301) 619-7808. For licensing issues, Dr. Paul Mele, Office of Research & Technology Assessment, (301) 619-6664, bh at telefax (301) 619-5034.

**SUPPLEMENTARY INFORMATION:** The following patents are available for licensing:

(1) U.S. Patent Application No.: 09/570,022.

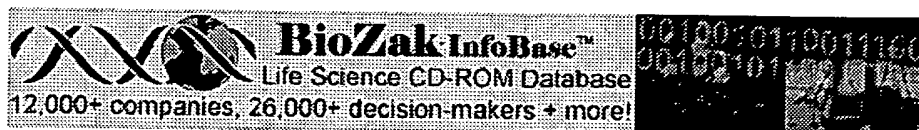
Filed: May 12, 2000.

Title: Previns as Specific Inhibitors and Therapeutic Agents for Botulinum Toxin B and Tetanus Neurotoxins.

Supplementary Information: The compounds of the invention may be used as molecular building blocks to create compounds that are optimized for inhibiting the protease activity of Botulinum B and tetanus toxins. Foreign rights (PCT/US00/13215) are also available.

(2) U.S. Patent Application No.: 09/570,023.

Filed: May 12, 2000.



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**Notice:** Availability for Non-Exclusive, Exclusive, or Partially Ex  
Licensing of U.S. Patent Application Concerning Microfluidized  
Leishmania Lysate and Methods of Making and Using Thereof

**Career  
Opportunities**

**Federal Register:** August 5, 2002 (Volume 67, Number 150)  
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**AGENCY:** Department of the Army, DOD.

**Daily News  
Highlights**

**ACTION:** Notice.

**Federal Register**

**SUMMARY:** In accordance with 37 CFR 404.6 and 404.7, announcement i  
made of the availability for licensing of U.S. Patent Application  
09/975,020 entitled "Microfluidized Leishmania Lysate and Methods  
Making and Using Thereof," filed October 12, 2001. Foreign rights  
(PCT/US01/31894) are also available. The United States Government,  
represented by the Secretary of the Army has rights in this invent

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**ADDRESSES:** Commander, U.S. Army Medical Research and Materiel Comm  
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**FOR FURTHER INFORMATION CONTACT:** For patent issues, Ms. Elizabeth  
Arwine, Patent Attorney, (301) 619-7808. For licensing issues, Dr.  
Mele, Office of Research & Technology Assessment, (301) 619-6664,  
at telefax (301) 619-5034.

**Calendar**

**Technology  
Highlights**

**SUPPLEMENTARY INFORMATION:** This invention relates generally to  
microfluidized Leishmania lysate preparations. In particular, the  
present invention relates to microfluidized Leishmania lysate  
preparations for assays and immunogenic compositions.

**Web Links**

**CGMP Notes**

Luz D. Ortiz,  
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[FR Doc. 02-19715 Filed 8-2-02; 8:45 am]  
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Good Manufacturing Practices Production of **Leishmania** Skin test antigen: 2. Production of a **Microfluidized Lysate**.

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**Title Development of a Leishmania Skin Test Antigen (**

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# Optimisation of an ELISA for the serodiagnosis of visceral leishmaniasis using in vitro derived promastigote antigens

G. -Halli R. Rajasekariah<sup>a</sup>, Jeffrey R. Ryan<sup>b</sup>, Scott R. Hillier<sup>a</sup>, Lisa P. Yi<sup>b</sup>, John M. Stiteler<sup>b</sup>, Liwang Cui<sup>b</sup>, Anthony M. Smithyman<sup>a</sup> and Samuel K. Martin<sup>b</sup>

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Received 28 June 2000; revised 24 January 2001; accepted 25 January 2001 Available online 25 April 2001.


## Abstract

An antibody detection ELISA was developed for diagnosis of visceral leishmaniasis. Antigens released by *Leishmania donovani* promastigotes into a protein-free medium were used. SDS-PAGE analysis has indicated that Ld-ESM contain several protein antigens. Titration and checker-board analyses were performed to optimise the assay protocol. Optimal results were obtained when antigen (50  $\mu$ g/ml) was coated with PBS-methyl glyoxal buffer, and wells blocked with 0.5% casein. A serum dilution of 1:500 in antigen-coated wells, blocked with 0.5% casein, generated lowest absorbance with Ref-ve sera and higher absorbance with Ref+ve sera. All steps of the ELISA were performed at room temperature. The S/N ratio, the differential absorbance between the negative sample vs. the test or Ref+ve sample, was used to quantify the specific antigen and antibody reactions. An anti-human monoclonal antibody conjugated with HRP (MAb-conjugate) outperformed a commercially available anti-human polyclonal antibody conjugate (PAb-conjugate). The MAb-conjugate gave minimal background reactions with endemic sera. Optimised final assay steps mentioned below were used to evaluate sera samples from field trials. ELISA wells were coated with 50  $\mu$ g/ml Ld-ESM mixed in PBS-methyl glyoxal overnight, and after removing the antigen, blocked with 0.5% casein for 1 h at RT. Patient sera along with control sera, diluted to 1:500 in PBS/T, were reacted for 1 h at RT. After washing the plate with PBS/T, wells were reacted

with MAb-conjugate for 40 min at RT, and after washing, binding of antibodies was visualized by using TMB as a chromogen substrate. The relative specific binding was quantified by the S/N ratio. A batch of  $n=22$  endemic sera from North Africa were evaluated and resulted with 100% specificity and sensitivity, 99.99% PPV and 95.45% NPV. The specificity and sensitivity of this assay will be further evaluated in planned retrospective and prospective multi-site trials.

**Author Keywords:** Anti-human HRP conjugate; ELISA; Endemic sera; ESM antigen; Field-ELISA; *Leishmania donovani*; Promastigote; Specific antibody reactivity; Signal/noise ratio; Visceral leishmaniasis

**Abbreviations:** ELISA, enzyme-linked immunosorbent assay; Ld-Ab f-ELISA, *L. donovani* antibody field ELISA; Ld-ESM, excretory, secretory and metabolic antigens released by *L. donovani* promastigotes during in vitro maintenance in serum-free and protein-free medium; MAb-conjugate, mouse anti-human monoclonal IgG–HRP conjugate; NPV, negative predictive value; PAb-conjugate, goat anti-human polyclonal IgG–HRP conjugate; PBS–methyl glyoxal, phosphate buffer saline supplemented with 1% methyl glyoxal; PBS/T, phosphate buffered saline supplemented with 0.05% Tween 20; PPV, positive predictive value; TMB, tetramethylbenzidine; Ref-ve sera, defined negative sera, not exposed to *L. donovani* infection; Ref+ve sera, defined sera from patients infected with *L. donovani* parasites; S/N Ratio, signal noise ratio, i.e., the differential absorbance between the Ref+ve and Ref-ve sera; RT, room temperature, the ambient temperature used to mimic the field situation; VL, visceral leishmaniasis due to *L. donovani*

 Corresponding author. Tel.: +61-2-9905-0133; fax: +61-2-9905-6426; email: [admin@cellabs.com.au](mailto:admin@cellabs.com.au)

## Journal of Immunological Methods

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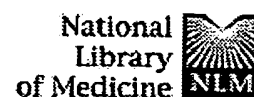
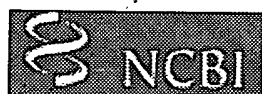
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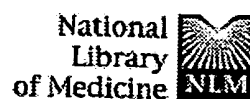
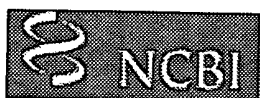
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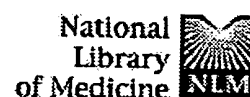
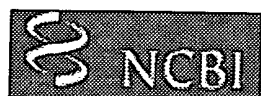
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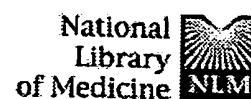
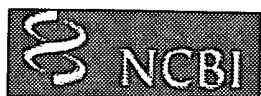
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An antibody detection ELISA was developed for diagnosis of visceral leishmaniasis. Antigens released by *Leishmania donovani* promastigotes into a protein-free medium were used. SDS-PAGE analysis has indicated that Ld-ESM contain several protein antigens. Titration and checker-board analyses were performed to optimise the assay protocol. Optimal results were obtained when antigen (50 microg/ml) was coated with PBS-methyl glyoxal buffer, and wells blocked with 0.5% casein. A serum dilution of 1:500 in antigen-coated wells, blocked with 0.5% casein, generated lowest absorbance with Ref-ve sera and higher absorbance with Ref+ve sera. All steps of the ELISA were performed at room temperature. The S/N ratio, the differential absorbance between the negative sample vs. the test or Ref+ve sample, was used to quantify the specific antigen and antibody reactions. An anti-human monoclonal antibody conjugated with HRP (MAb-conjugate) outperformed a commercially available anti-human polyclonal antibody conjugate (PAb-conjugate). The MAb-conjugate gave minimal background reactions with endemic sera. Optimised final assay steps mentioned below were used to evaluate sera samples from field trials. ELISA wells were coated with 50 microg/ml Ld-ESM mixed in PBS-methyl glyoxal overnight, and after removing the antigen, blocked with 0.5% casein for 1 h at RT. Patient sera along with control sera, diluted to 1:500 in PBS/T, were reacted for 1 h at RT. After washing the plate with PBS/T, wells were reacted with MAb-conjugate for 40 min at RT, and after washing, binding of antibodies was visualized by using TMB as a chromogen substrate. The relative specific binding was quantified by the S/N ratio. A batch of n=22 endemic sera from North Africa were evaluated and resulted with 100% specificity and sensitivity, 99.99% PPV and 95.45% NPV. The specificity and sensitivity of this assay will be further evaluated in planned retrospective and prospective



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### **The direct agglutination test for diagnosis of visceral leishmaniasis under field conditions in Sudan: comparison of aqueous and freeze-dried antigens.**

**Zijlstra EE, Osman OF, Hofland HW, Oskam L, Ghalib HW, el-Hassan AM, Kager PA, Meredith SE.**

Department of Infectious Diseases, Tropical Medicine and AIDS, Academic Medical Centre, Amsterdam, The Netherlands.

Related Resources

The performance of the direct agglutination test (DAT) was evaluated under field conditions in an endemic area of visceral leishmaniasis in eastern Sudan, using aqueous (Aq) antigen which has to be kept refrigerated and a newly developed freeze-dried (FD) antigen which is stable at ambient temperature. Both antigens compared well, with 92-98% of readings being identical or only with one dilution difference in titre. FD antigen gave titres that were identical with Aq antigen in 73% of samples, higher in 19%, and lower in 8%. Owing to high ambient temperatures and low humidity, microtitre plate wells dried out during the standard procedures for elution and incubation. However, shortening the elution time from 12 to 4 h proved possible for both antigens; incubation could be reduced from 24 to 10 h for Aq antigen, after which the plates could still be read. Incubation with FD antigen required 18 h and the plates needed to be kept cool because of evaporation. Despite the longer procedure with the FD antigen, the DAT can be completed in 24 h and the use of this stable antigen, that does not require refrigeration, is a major improvement in performing the DAT under unfavourable field conditions.

PMID: 9509176 [PubMed - indexed for MEDLINE]

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**Title:** From genomes to vaccines: *Leishmania* as a model

**Author(s):** Renata Almeida ; Alan Norrish ; Mark Levick ; David Vetrie ; Tom Freeman ; Jaak Vilo ; Alasdair Ivens ; Uta Lange ; Carmel Stober ; Sharon McCann ; Jenefer M. Blackwell

**Source:** Philosophical Transactions: Biological Sciences **Volume:** 357 **Number:** 1417 **Page:** 5 – 11

**DOI:** 10.1098/rstb.2001.0985

**Publisher:** The Royal Society

**Abstract:** The 35 Mb genome of *Leishmania* should be sequenced by late 2002. It contains approximately 8500 genes that will probably translate into more than 10 000 proteins. In the laboratory we have been piloting strategies to try to harness the power of the genome-proteome for rapid screening of new vaccine candidate. To this end, microarray analysis of 1094 unique genes identified using an EST analysis of 2091 cDNA clones from spliced leader libraries prepared from different developmental stages of *Leishmania* has been employed. The plan was to identify amastigote-expressed genes that could be used in high-throughput DNA-vaccine screens to identify potential new vaccine candidates. Despite the lack of transcriptional regulation that polycistronic transcription in *Leishmania* dictates, the data provide evidence for a high level of post-transcriptional regulation of RNA abundance during the developmental cycle of promastigotes in culture and in lesion-derived amastigotes of *Leishmania major*. This has provided 147 candidates from the 1094 unique genes that are specifically upregulated in amastigotes and are being used in vaccine studies. Using DNA vaccination, it was demonstrated that pooling strategies can work to identify protective vaccines, but it was found that some potentially protective antigens are masked by other disease-exacerbatory antigens in the pool. A total of 100 new vaccine candidates are currently being tested separately and in pools to extend this analysis, and to facilitate retrospective bioinformatic analysis to develop predictive algorithms for sequences that constitute potentially protective antigens. We are also working with other members of the *Leishmania* Genome Network to determine whether RNA expression determined by microarray analyses parallels expression at the protein level. We believe we are making good progress in developing strategies that will allow rapid translation of the sequence of *Leishmania* into potential interventions for disease control in humans.

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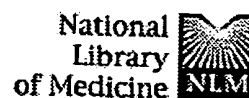
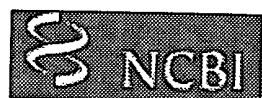
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## An overview of paediatric leishmaniasis.

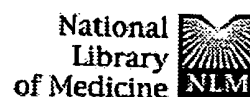
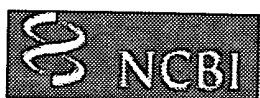
**Kafetzis DA.**

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University of Athens Second Department of Paediatrics, "P. and A. Kyriakou" Children's Hospital, Athens, 11527 Greece.  
kafetzis@ath.forthnet.gr

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Leishmaniasis, a parasitic disease transmitted by the bite of some species of sandflies affects various age groups depending on the infecting *Leishmania* species, geographic location, disease reservoir, and host immunocompetence. Visceral leishmaniasis is the most severe form of the disease affecting children. The extent and presentation of the disease depend on several factors, including the humoral and cell-mediated immune response of the host, the virulence of the infecting species, and the parasite burden. Children are at greater risk than adults in endemic areas. Malnutrition contributes to the development of disease, and incomplete therapy of initial disease is a risk factor for recurrence of leishmaniasis. Children usually present with intermittent fever, paleness, refusal to feed or anorexia, weight loss, and abdominal distension. Splenomegaly, hepatomegaly, lymph node enlargement, thrombocytopaenia, anaemia, leukopaenia and hypergammaglobulinemia are the most common findings in Paediatric leishmaniasis. Molecular methods appear to offer the promise of accurate non-invasive tools for the diagnosis of Leishmaniasis. Till these methods are evaluated, definite diagnosis will rely on the demonstration of the infecting parasite in various tissues. World-wide, with the notable exception of India, pentavalent antimonial compounds remain the most effective and the most affordable therapy for this disease. Lipid formulations of amphotericin B were assessed as short duration treatment and were proved to be effective. However, their cost precludes their wide use in developing countries. Miltefosine, a new oral agent, might prove effective, safe, and affordable. Strategies aimed at control of the micro-population of sandflies, eradication of canine leishmaniasis, and offering personal protection against sandfly bites, together with health education programs in developing countries, can help control the disease. Development of an effective vaccine remains a priority.



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### Identification and purification of immunogenic proteins from nonliving promastigote polyvalent Leishmania vaccine (Leishvacin ).

Cardoso SR, da Silva JC, da Costa RT, Mayrink W, Melo MN, Michalick MS, Liu IA, Fujiwara RT, Nascimento E.

Departamento de Parasitologia, Instituto de Ciencias Biologicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil.

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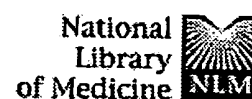
Immunogenic proteins from nonliving promastigote polyvalent Leishmania vaccine against American tegumentary leishmaniasis (Leishvacin ), produced by BioBr s (Biochemistry of Brazil ), Montes Claros, State of Minas Gerais, Brazil, were identified and purified by polyacrylamide electrophoresis gel and electroelution. C57BL/10 mice were vaccinated with proteins with estimated molecular weights of 42, 46, 63, 66, 73, 87, 97, and 160kDa in three doses of 30 g of each protein at 15-day intervals combined with 250 microg of Corynebacterium parvum followed by a challenge infection with 10(5) infective promastigotes from Leishmania (Leishmania) amazonensis. The ability of these proteins to induce immune response and protection was analyzed. No statistical difference was observed in the level of IFN-gamma induced by proteins in vaccinated groups in comparison with control groups. Six months after challenge infection, protection levels of 28.57; 42.86; 57.14; 42.86; 42.86, 57.14; 42.86 and 57.14% were demonstrated for each purified protein.

PMID: 12806454 [PubMed - in process]

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## Recent developments in leishmaniasis.

Melby PC.

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Medical Service, Department of Veterans Affairs Medical Center, South Texas Veterans Health Care System, USA. [melby@uthscsa.edu](mailto:melby@uthscsa.edu)

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**PURPOSE OF REVIEW:** The leishmaniasis, caused by protozoan parasites of the genus *Leishmania*, are a significant health problem in many regions of the world. This review highlights the recent advances in the study of leishmaniasis related to parasite biology, disease pathogenesis, clinical evaluation and treatment, and prevention. **RECENT FINDINGS:** Genetic heterogeneity and clonal diversity is common among *Leishmania* strains. Gene knockout, overexpression, and re-introduction studies have identified a number of genes that play a role in parasite virulence. Surprisingly, the importance of the surface lipophosphoglycan in parasite virulence appears to differ among *Leishmania* spp. Studies in experimental animal models have further defined the roles of CD4 and CD8 T cells, IL-4, IL-10, and IL-12 in the control, maintenance, or progression of disease. The effect of *Leishmania* on dendritic cells and macrophage effector function has also been an important area of investigation. A number of new vaccine candidates have been identified through experimental animal studies. Clinical studies of leishmaniasis have focused on the host determinants of disease (most notably HIV co-infection), serological and DNA-based diagnostic assays, and treatment. Antimony-resistant cases of cutaneous and visceral leishmaniasis have become more common; liposomal amphotericin and oral miltefosine are promising alternative therapies. **SUMMARY:** Significant advances have been made in the areas of pathogenesis, host defence, and treatment of leishmaniasis. A number of new vaccine candidates and potential targets of drug therapy have been identified, but progress from preclinical studies to clinical trials has been slow. Translational research, built upon the solid foundation of existing and ongoing basic investigation, is a high priority.

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**Study of the safety, immunogenicity and efficacy of attenuated and killed *Leishmania* (*Leishmania*) major vaccines in a rhesus monkey (*Macaca mulatta*) model of the human disease.**

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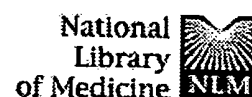
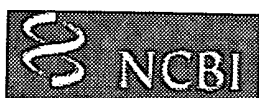
Amaral VF, Teva A, Oliveira-Neto MP, Silva AJ, Pereira MS, Cupolillo E, Porrozzzi R, Coutinho SG, Pirmez C, Beverley SM, Grimaldi G Jr.

Departamento de Imunologia, Instituto Oswaldo Cruz-Fiocruz, Rio de Janeiro, Brasil.

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We have compared the efficacy of two *Leishmania* (*Leishmania*) major vaccines, one genetically attenuated (DHFR-TS deficient organisms), the other inactivated [autoclaved promastigotes (ALM) with bacillus Calmete-Guerin (BCG)], in protecting rhesus macaques (*Macaca mulatta*) against infection with virulent *L. (L.) major*. Positive antigen-specific recall proliferative response was observed in vaccinees (79% in attenuated parasite-vaccinated monkeys, versus 75% in ALM-plus-BCG-vaccinated animals), although none of these animals exhibited either augmented in vitro gamma interferon (IFN-gamma) production or positive delayed-type hypersensitivity (DTH) response to the leishmanin skin test prior to the challenge. Following challenge, there were significant differences in blastogenic responses ( $p < 0.05$ ) between attenuated-vaccinated monkeys and naive controls. In both vaccinated groups very low levels of antibody were found before challenge, which increased after infective challenge. Protective immunity did not follow vaccination, in that monkeys exhibited skin lesion at the site of challenge in all the groups. The most striking result was the lack of pathogenicity of the attenuated parasite, which persisted in infected animals for up to three months, but were incapable of causing disease under the conditions employed. We concluded that both vaccine protocols used in this study are safe in primates, but require further improvement for vaccine application.

PMID: 12471434 [PubMed - indexed for MEDLINE]



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## Vaccination against Leishmania infections.

**Mauel J.**

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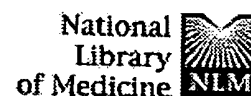
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Leishmaniasis, that affects millions of people worldwide, is an infectious disease caused by the protozoan parasite *Leishmania*. Incidence of the condition appears to be increasing in several parts of the world. Of the three main presentations of the disease, i.e. cutaneous, mucocutaneous and visceral, only the first one tends to heal spontaneously, while the other two are considered fatal if left to run their natural course. Recovery from leishmaniasis, whether spontaneous or drug-induced, is usually accompanied by solid immunity against reinfection, which provides a rationale for attempting to design vaccines against the disease. This review presents an outline of the main immunological features of *Leishmania* infections and of the mechanisms thought to operate in recovery from the disease. It describes various experimental approaches to vaccination in man and animal models, including the use of virulent and avirulent organisms, of dead parasites and extracts thereof, and of purified parasite proteins. Assays using novel technologies, such as the direct injection of DNAs encoding parasite proteins, or the inoculation of viral or bacterial vectors expressing such molecules, as well as recent experiments aimed at inducing an immune response against saliva of the insect vector, are also reviewed. Observations made during the course of these studies have reinforced the notion that vaccination against leishmaniasis is indeed feasible. However, in spite of intensive efforts by many groups and many reports of success in man and in animal models, a consensus is yet to emerge as to what constitutes the best approach to vaccination against leishmaniasis.

### Publication Types:

- Review
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## Vaccination against cutaneous leishmaniasis: current status.

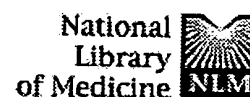
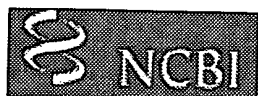
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Department of Veterans Affairs Medical Center, Medical Service, South Texas Veterans Health Care System, San Antonio, TX 78229, USA.  
melby@uthscsa.edu

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The different cutaneous leishmaniasis are distinct in their etiology, epidemiology, transmission, and geographical distribution. In most instances cutaneous leishmaniasis is limited to one or a few skin ulcers that develop at the site where the parasites were deposited during the bite of the sandfly vector. Lesions typically heal spontaneously after several months but some lesions can be large and follow a chronic, more severe course. Protective immunity is usually acquired following cutaneous infection with *Leishmania* spp., so prevention of disease through prophylactic immunization appears to be feasible. Since vaccination with live, virulent parasites is associated with an unacceptable rate of adverse events, attention has turned to the use of killed or attenuated parasite vaccines and defined subunit vaccines. Whole parasite vaccines have the advantage of delivering multiple antigenic epitopes that may be necessary for initiation of a broad-based immune response. Persistent or repeated immune-stimulation by parasite antigens and/or sustained expression of interleukin-12 appear to be critical elements in the development of durable immunity. A number of purified or recombinant antigens, when co-administered with a vaccine adjuvant, appear promising as vaccine candidates against cutaneous leishmaniasis. The sustained expression of recombinant *Leishmania* antigens by vaccination with DNA is an attractive approach because it mimics the persistent antigenic stimulation of subclinical infection. Effective vaccine-induced immunity must generate an antigen-specific memory T cell population that, upon exposure to the infecting parasite, rapidly produces a type 1 effector T cell response that leads to interferon-gamma-mediated activation of infected macrophages to kill the intracellular parasites. This parasite-directed recall response must be prompt and of sufficient magnitude to overcome the subversive effect that the intracellular infection has on macrophage effector function. It is unlikely that vaccination against cutaneous leishmaniasis would induce sterile immunity, but a small number of parasites are likely to persist subclinically.



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**Vaccination of C57BL/10 mice against cutaneous Leishmaniasis using killed promastigotes of different strains and species of Leishmania.**

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Mayrink W, Santos GC, Toledo Vde P, Guimaraes TM, Machado-Coelho GL, Genaro O, da Costa CA.

Departamento de Parasitologia, Instituto de Ciencias Biologicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG.

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Antigenic extracts from five Leishmania stocks were used to vaccinate C57BL/10 mice. The Leishvacin(R) and PH8 monovalent vaccine yielded the highest IFN-gamma levels in the supernatants of spleen cell culture from vaccinated animals. Each single strain immunized group showed evidence of protective immunity six months after the challenge with promastigotes of Leishmania (Leishmania) amazonensis. No differences were detected between the vaccinated groups. It can be concluded that vaccines composed of single Leishmania stocks can provide protection to C57BL/10 mice against L. (L.) amazonensis infection.

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*Current Opinion in Infectious Diseases* 2002; 15(5):485-490

# Recent developments in leishmaniasis

Peter C. Melby

## Purpose of review

The leishmaniasis, caused by protozoan parasites of the genus *Leishmania*, are a significant health problem in many regions of the world. This review highlights the recent advances in the study of leishmaniasis related to parasite biology, disease pathogenesis, clinical evaluation and treatment, and prevention.

## Recent findings

Genetic heterogeneity and clonal diversity is common among *Leishmania* strains. Gene knockout, overexpression, and re-introduction studies have identified a number of genes that play a role in parasite virulence. Surprisingly, the importance of the surface lipophosphoglycan in parasite virulence appears to differ among *Leishmania* spp. Studies in experimental animal models have further defined the roles of CD4 and CD8 T cells, IL-4, IL-10, and IL-12 in the control, maintenance, or progression of disease. The effect of *Leishmania* on dendritic cells and macrophage effector function has also been an important area of investigation. A number of new vaccine candidates have been identified through experimental animal studies. Clinical studies of leishmaniasis have focused on the host determinants of disease (most notably HIV co-infection), serological and DNA-based diagnostic assays, and treatment. Antimony-resistant cases of cutaneous and visceral leishmaniasis have become more common; liposomal amphotericin and oral miltefosine are promising alternative therapies.

## Summary

Significant advances have been made in the areas of pathogenesis, host defence, and treatment of leishmaniasis. A number of new vaccine candidates and potential targets of drug therapy have been identified, but progress from preclinical studies to clinical trials has been slow. Translational research, built upon the solid foundation of existing and ongoing basic investigation, is a high priority.

**Keywords** immunity; *Leishmania*; leishmaniasis; sand fly; treatment; vaccines

## Abbreviations

CL cutaneous leishmaniasis

NO nitric oxide

**PKDL** post-kala azar dermal leishmaniasis

**PCR** polymerase chain reaction

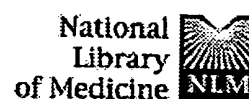
**Th** T helper

**VL** visceral leishmaniasis

Medical Service, Department of Veterans Affairs Medical Center, South Texas Veterans Health Care System, and  
Departments of Medicine and Microbiology, The University of Texas Health Science Center, San Antonio, Texas, USA

Correspondence to Peter C. Melby, MD, Department of Medicine, Division of Infectious Diseases, The University of Texas  
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Current Opinion in Infectious Diseases 2002; 15(5):485-490  
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## Vaccines for parasitic and bacterial diseases.

Reed SG, Campos-Neto A.

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Infectious Disease Research Institute, 1124 Columbia Street, Suite 600,  
98104, Seattle, WA, USA

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The first decade of the millennium should mark the beginning of a new era in vaccine development, reaping the rewards of advances in genome characterization, antigen identification, understanding the molecular bases of protective immune responses, and adjuvant design and development. Advances in all of these areas have culminated in vaccine candidates entering clinical testing. These include vaccines against two of humankind's oldest and deadliest diseases, tuberculosis and malaria. Several vaccine candidates for each of these diseases will be tested in humans during the next few years. A candidate vaccine for leishmaniasis, an infection that has taught us much about T-cell regulation of protection and disease in animal models, has been developed and is now in the clinic. There are indications both in animal models and in patients that vaccines may be used not only to protect but also to treat leishmania infections.

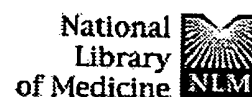
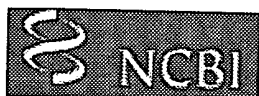
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## Development of a leishmaniasis vaccine: the importance of MPL.

Reed SG, Coler RN, Campos-Neto A.

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Corixa Corporation, Seattle, WA, USA.

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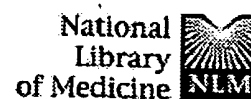
The parasite *Leishmania* has been used for pioneering work to define T-cell subsets and cytokine patterns mediating susceptibility or resistance to infectious pathogens. This understanding has been essential for the development of a new generation of candidate vaccines for major diseases, such as leishmaniasis themselves, tuberculosis and others. It is clear that effective vaccines can be developed through a combination of both antigen and adjuvant selection. Until recently, no adjuvants acceptable for use in human T-cell vaccines were available. However, one such adjuvant, monophosphoryl lipid A, has been shown to be safe and effective. Just as the understanding of T-cell responses has been necessary for the development of a new generation of vaccines, an understanding of signaling by antigen-presenting cells has been essential for adjuvant selection. A combination of antigens and an adjuvant that is effective at promoting durable T-helper 1 responses and is safe for human use comprise a promising vaccine candidate, Leish-111f. This vaccine has potential application in both the prevention and treatment of leishmaniasis.

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## Leishmania mexicana: chemistry and biochemistry of sodium stibogluconate (Pentostam).

**Berman JD, Grogl M.**

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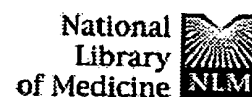
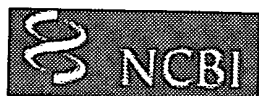
The chemical properties of the primary antileishmanial agent sodium stibogluconate (Pentostam), and the interaction of Pentostam with *Leishmania mexicana* amastigotes, have been investigated with the aid of [<sup>125</sup>Sb]Pentostam. The molecular weight by P2 chromatography showed [<sup>125</sup>Sb]Pentostam to be of multiple species of MW = 100-4000 Da, rather than the one species of 746 Da predicted by the commonly hypothesized structural formula. Nonradioactive Pentostam had a lower osmolality (789 mOsm for a 100 mg Sb/ml solution) than predicted (1644 mOsm), which indicates that the multiple components of Pentostam (Sb and derivatives of gluconic acid) are more closely complexed with each other than previously thought. When incubated with *L. mexicana* amastigotes, labeled drug was bound to at least six polypeptides of molecular weights ranging from 14,000 to 68,000 Da as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Interaction with the polypeptides is presumed to contribute to the antileishmanial action of Pentostam.

PMID: 2844580 [PubMed - indexed for MEDLINE]

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**Isolation of *Leishmania mexicana* (Kinetoplastida: Trypanosomatidae) from *Lutzomyia anthophora* (Diptera: Psychodidae) collected in Texas.**

PubMed Services

**McHugh CP, Grogl M, Kreutzer RD.**

Occupational and Environmental Health Directorate, Armstrong Laboratory, Brooks Air Force Base, TX 78235.

Related Resources

Three of 27 female *Lutzomyia anthophora* (Addis) collected in Texas from the nest of a southern plains woodrat, *Neotoma micropus* Baird, during October 1991 were infected with flagellate protozoans. Isolates were grown in Schneider's *Drosophila* medium supplemented with 20% fetal bovine serum, and isozyme analysis of two of the isolates determined the parasites to be *Leishmania mexicana* (Biagi). These are the first isolations of *Leishmania* from field-collected sand flies in North America north of Mexico. Possible reasons for the lack of human cases near the focus are presented.

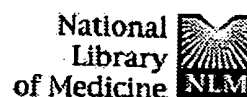
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☐ 1: Exp Parasitol. 1996 Dec;84(3):400-9.[Related Articles, Links](#)

### **Leishmania spp: temperature sensitivity of promastigotes in vitro as a model for tropism in vivo.**

PubMed Services

**Callahan HL, Portal IF, Bensinger SJ, Grogl M.**

American Consulate-Rio de Janeiro, U.S. Army Medical Research Unit-Brazil.

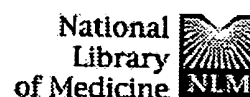
Related Resources

Since in humans, skin temperature is lower than internal temperature, the temperature sensitivity of *Leishmania* may influence the tropism of *Leishmania* in the human host; temperature-sensitive parasites may remain in the skin, temperature-resistant parasites may go to the viscera. In order to pursue the genetic factors controlling *Leishmania* tropism, we have developed an in vitro promastigote temperature model. Promastigote growth is measured at 30, 32, and 34 degrees C and compared with growth at the control temperature (25 degrees C). The results from tests of the promastigote temperature sensitivity of eight species (33 different strains) show that visceral species (*L. donovani* and *L. chagasi*) are more temperature resistant than cutaneous species (*L. major*, *L. tropica*, *L. mexicana*, *L. braziliensis*, *L. panamensis*, and *L. amazonensis*), that Old World species are more temperature-resistant than New World species, and that within the New World cutaneous species there are three distinct temperature sensitivity groupings (*L. mexicana* > *L. braziliensis* and *L. panamensis* > *L. amazonensis*). Interestingly, viscerotropic *L. tropica* from Operation Desert Storm and *L. donovani* complex strains isolated from cutaneous lesions are more and less temperature-sensitive, respectively, than strains of the same species with the expected tropism in vivo.

PMID: 8948329 [PubMed - indexed for MEDLINE]

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☐ 1: Antimicrob Agents Chemother. 1997  
Apr;41(4):818-22.

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### An axenic amastigote system for drug screening.

PubMed Services

Callahan HL, Portal AC, Devereaux R, Grogl M.

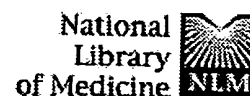
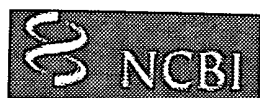
U.S. Army Medical Research Unit-Brazil.

Heather\_Callahan@WRSMTP-CCMAIL.ARMY.MIL

Related Resources

Currently available primary screens for selection of candidate antileishmanial compounds are not ideal. The choices include screens that are designed to closely reflect the situation in vivo but are labor-intensive and expensive (intracellular amastigotes and animal models) and screens that are designed to facilitate rapid testing of a large number of drugs but do not use the clinically relevant parasite stage (promastigote model). The advent of successful in vitro culture of axenic amastigotes permits the development of a primary screen which is quick and easy like the promastigote screen but still representative of the situation in vivo, since it uses the relevant parasite stage. We have established an axenic amastigote drug screening system using a *Leishmania mexicana* strain (strain M379). A comparison of the 50% inhibitory concentration (IC<sub>50</sub>) drug sensitivity profiles of M379 promastigotes, intracellular amastigotes, and axenic amastigotes for six clinically relevant antileishmanial drugs (sodium stibogluconate, meglumine antimoniate, pentamidine, paromomycin, amphotericin B, WR6026) showed that M379 axenic amastigotes are a good model for a primary drug screen. Promastigote and intracellular amastigote IC<sub>50</sub>s differed for four of the six drugs tested by threefold or more; axenic amastigote and intracellular amastigote IC<sub>50</sub>s differed by twofold for only one drug. This shows that the axenic amastigote susceptibility to clinically used reference drugs is comparable to the susceptibility of amastigotes in macrophages. These data also suggest that for the compounds tested, susceptibility is intrinsic to the parasite stage. This contradicts previous hypotheses that suggested that the activities of antimonial agents against intracellular amastigotes were solely a function of the macrophage.

PMID: 9087496 [PubMed - indexed for MEDLINE]



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☐ 1: J Parasitol. 1999 Apr;85(2):354-9.[Related Articles, Links](#)

### Successful topical treatment of murine cutaneous leishmaniasis with a combination of paromomycin (Aminosidine) and gentamicin.

PubMed Services

Grogl M, Schuster BG, Ellis WY, Berman JD.

Department of Biologics Research, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D.C. 20307-5100, USA.

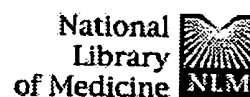
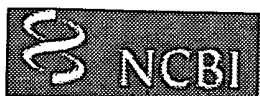
Related Resources

Cutaneous leishmaniasis is presently treated with 20 days of parenteral therapy with a frequently toxic drug (antimony). Topical formulations of paromomycin (15%) plus methylbenzethonium chloride (MBCL, 12%) or plus urea (10%) in soft white paraffin have been tested for Old and New World disease in humans. We compared the efficacy of a new topical formulation, WR 279,396 (paromomycin [15%] plus gentamicin [0.5%]) to the clinical formulations in the treatment of cutaneous disease in BALB/c mice. Sixty-day-old lesions were treated twice a day for 10 days, and the response to therapy was determined over a further 70 days. For ulcers due to *Leishmania major* or to *Leishmania mexicana*, 100% of lesions in the WR 279,396 group healed by day 20 after therapy and did not relapse by day 70; 83% of lesions healed without relapse in the paromomycin-MBCL group. In the paromomycin-urea group, 100% of *L. major* lesions healed by day 30 but 30% relapsed. For ulcers due to *Leishmania panamensis* or *Leishmania amazonensis*, all lesions treated with WR 279,396 healed and did not relapse; < 50% of lesions treated with paromomycin-MBCL healed by day 30, and all lesions relapsed by day 70. In addition to being active, WR 279,396 was not toxic in this model and appears to have a cosmetic effect (promoting hair growth, healing, and limiting the size of the scar).

PMID: 10219319 [PubMed - indexed for MEDLINE]

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☐ 1: Lab Anim Sci. 1999 Oct;49(5):519-21.[Related Articles, Links](#)

### Comparison of adjuvants with *Leishmania* antigens in a guinea pig model to induce delayed-type hypersensitivity responses.

PubMed Services

Briand EJ, Ruble GR, Stiteler J, Harris LD, Burge JR, Soranaka ET, Glenn G, Quance-Fitch F, Rowton ED.

Walter Reed Army Institute of Research, Washington, DC, USA.

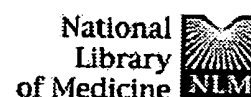
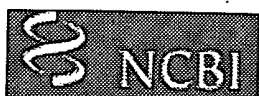
Related Resources

**BACKGROUND AND PURPOSE:** Guinea pigs have been a traditional model for studies of delayed-type hypersensitivity. They are the natural host of *Leishmania enriettii* and have been experimentally infected with other species of *Leishmania*. They have been used as a skin-test model to screen potential antigens for use in diagnostic tests for *Leishmania*. Use of complete Freund's adjuvant (CFA), along with whole promastigote *Leishmania* antigen, was necessary to sensitize guinea pigs to invoke a sufficient cell-mediated immune response. However, use of CFA has come under scrutiny by Animal Care and Use Committees due to the pathologic changes associated with its use. **METHODS:** Thirty-two specific-pathogen-free male Hartley guinea pigs were inoculated with *Leishmania* antigens alone or mixed with one of three adjuvants (CFA, TiterMax, and liposomes), and were skin tested 2 weeks later. **RESULTS:** For the *Leishmania* antigens tested, guinea pigs that received liposomes as an adjuvant had skin-test responses comparable to those of guinea pigs that received CFA. TiterMax was also tested, but cellular responses at antigen test sites were poor. **CONCLUSIONS:** Liposomes can be used in this model as a safe, effective adjuvant.

PMID: 10551453 [PubMed - indexed for MEDLINE]

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☐ 1: Vet Parasitol. 1986 Mar;20(1-3):195-215.[Related Articles, Links](#)

### Detection and characterization of Leishmania species and strains from mammals and vectors by hybridization and restriction endonuclease digestion of kinetoplast DNA.

PubMed Services

Jackson PR, Lawrie JM, Stiteler JM, Hawkins DW, Wohlhieter JA, Rowton ED.

Related Resources

Leishmania parasites from animals, man or insect vectors were characterized by the gel electrophoresis of restriction endonuclease enzyme-produced mitochondrial (kinetoplast) DNA (kDNA) fragments and/or by DNA-DNA hybridization with <sup>32</sup>P-labelled cloned, or uncloned, kDNA fragment probes from type isolates. The electrophoretic separation of kDNA fragments is a sensitive method for detecting genetic similarities and differences among Leishmania. Parasites with similar kDNA restriction fragment patterns belong to the same schizodeme and schizodeme analysis is useful for studying Leishmania populations. Cloned, species-specific kDNA probes detected Leishmania in sandflies and in liver, spleen or blood preparations from infected animals. Cloned DNA probes also hybridized to immobilized kDNA from in vitro cultivated promastigotes and detected as few as 100 parasites in a species-specific manner. Sensitive DNA hybridization probes should be useful in research on the immunology, chemotherapy or epidemiology of animal and human leishmaniasis.

PMID: 3010541 [PubMed - indexed for MEDLINE]

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## Journal of Immunological Methods

Volume 252, Issues 1-2, 1 June 2001, Pages 105-119

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# Optimisation of an ELISA for the serodiagnosis of visceral leishmaniasis using in vitro derived promastigote antigens

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Received 28 June 2000; revised 24 January 2001; accepted 25 January 2001 Available online 25 April 2001.


## Abstract

An antibody detection ELISA was developed for diagnosis of visceral leishmaniasis. Antigens released by *Leishmania donovani* promastigotes into a protein-free medium were used. SDS-PAGE analysis has indicated that Ld-ESM contain several protein antigens. Titration and checker-board analyses were performed to optimise the assay protocol. Optimal results were obtained when antigen (50 µg/ml) was coated with PBS-methyl glyoxal buffer, and wells blocked with 0.5% casein. A serum dilution of 1:500 in antigen-coated wells, blocked with 0.5% casein, generated lowest absorbance with Ref-ve sera and higher absorbance with Ref+ve sera. All steps of the ELISA were performed at room temperature. The S/N ratio, the differential absorbance between the negative sample vs. the test or Ref+ve sample, was used to quantify the specific antigen and antibody reactions. An anti-human monoclonal antibody conjugated with HRP (Mab-conjugate) outperformed a commercially available anti-human polyclonal antibody conjugate (PAb-conjugate). The Mab-conjugate gave minimal background reactions with endemic sera. Optimised final assay steps mentioned below were used to evaluate sera samples from field trials. ELISA wells were coated with 50 µg/ml Ld-ESM mixed in PBS-methyl glyoxal overnight, and after removing the antigen, blocked with 0.5% casein for 1 h at RT. Patient sera along with control sera, diluted to 1:500 in PBS/T, were reacted for 1 h at RT. After washing the plate with PBS/T, wells were reacted with Mab-conjugate for 40 min at RT, and after washing, binding of antibodies was visualized

by using TMB as a chromogen substrate. The relative specific binding was quantified by the S/N ratio. A batch of  $n=22$  endemic sera from North Africa were evaluated and resulted with 100% specificity and sensitivity, 99.99% PPV and 95.45% NPV. The specificity and sensitivity of this assay will be further evaluated in planned retrospective and prospective multi-site trials.

**Author Keywords:** Anti-human HRP conjugate; ELISA; Endemic sera; ESM antigen; Field-ELISA; *Leishmania donovani*; Promastigote; Specific antibody reactivity; Signal/noise ratio; Visceral leishmaniasis

**Abbreviations:** ELISA, enzyme-linked immunosorbent assay; Ld-Ab f-ELISA, *L. donovani* antibody field ELISA; Ld-ESM, excretory, secretory and metabolic antigens released by *L. donovani* promastigotes during in vitro maintenance in serum-free and protein-free medium; MAb-conjugate, mouse anti-human monoclonal IgG-HRP conjugate; NPV, negative predictive value; PAb-conjugate, goat anti-human polyclonal IgG-HRP conjugate; PBS-methyl glyoxal, phosphate buffer saline supplemented with 1% methyl glyoxal; PBS/T, phosphate buffered saline supplemented with 0.05% Tween 20; PPV, positive predictive value; TMB, tetramethylbenzidine; Ref-ve sera, defined negative sera, not exposed to *L. donovani* infection; Ref+ve sera, defined sera from patients infected with *L. donovani* parasites; S/N Ratio, signal noise ratio, i.e., the differential absorbance between the Ref+ve and Ref-ve sera; RT, room temperature, the ambient temperature used to mimic the field situation; VL, visceral leishmaniasis due to *L. donovani*

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Volume 252, Issues 1-2, 1 June 2001, Pages 105-119

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